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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Glycosphingolipids (GSLs) are fundamental components of all organisms, yet their functions remain largely unknown. In addition to immunological functions, these molecules regulate the "social behavior" of cells, including the cellular development of the nervous system, oncological transformation and metastasis. Some GSLs may be useful in the treatment of neurological disorders, including peripheral nerve injury as well as central neuropathies. The glucosyl ceramide synthase inhibitor (1R,2R)-(+)-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), has been shown to be a useful lead compound for the disruption of GSL expression to examine the effect of an altered GSL ensemble on nerve cell development. Despite their low phylogenetic status, insect nerve cells mimic the complexity displayed by the much more complicated human brain and nervous system to an amazing degree. The insect *Manduca sexta* provides a simplified developmental model which will allow examination of the effects of the PDMP analogs. An important aspect is the isolation and identification of the insect GSLs, so that the GSL expression can be correlated with neuronal development to unravel important structure-function relationships of GSLs. Insights from this insect model will facilitate the design and synthesis of new drugs to promote the rapid healing of diverse injuries and treatment of neuronal defects.

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Introduction

This project consists of 3 interconnected aims: 1) The synthesis of novel PDMP analogs, which are capable of altering GSL expression. 2) Structural determination of the GSL content in *Manduca sexta*. Ultimately, we will be able to examine the effects of the PDMP analogs on the ensemble of expressed GSL's. 3) Examination of the biological effects of the PDMP analogs on *Manduca sexta*. Ultimately, this will involve studies performed at the cellular level (cell culture experiments), tissue level (neuronal pathfinding experiments), and at the behavioral level (mating and feeding behaviors). These results will be compared with results from the *M. sexta* GSL extraction experiments in order to correlate specific molecular changes with specific biological effects ("PDMP" —> Alteration of GSL Expression —> Biological Effect in *M. sexta*). The 1st and 2nd parts of the project are being performed in the Polt Group laboratory in the Chemistry Department at the U. of A. The 3rd part is being performed in the Hildebrand Group laboratory in the Neuroscience Department at the U. of A. Insects, which lack a cellular immune system should show the modulatory effects of GSL's, uncomplicated by immunological responses.

Body

Chemical Synthesis of PDMP Analogs The synthesis of several new PDMP analogs using the published methodology[1] has been accomplished. (**Fig. 1**) Viewed in this way, the D-threo-propanolamine backbone common to all the glucosylceramide synthase inhibitors is simply a scaffold for 3 components of the pharmacophore. Note that we have retained the morpholino structure for the "Amine Moiety" in compounds **2**, **3**, **4**, and **5**, to provide a baseline for optimization of the "Aromatic Moiety" of the pharmacophore. Previous efforts to optimize the chain length of the "Amide Moiety" for biological activity results in a compromise between water solubility (shorter chains) and membrane insertion potential (longer chains). (**Fig. 2**) Thus, for simple amine and aromatic moieties, it seems that C_{10} is a reasonable length for cell culture experiments, and C_{16} is a reasonable length for *in vivo* work.

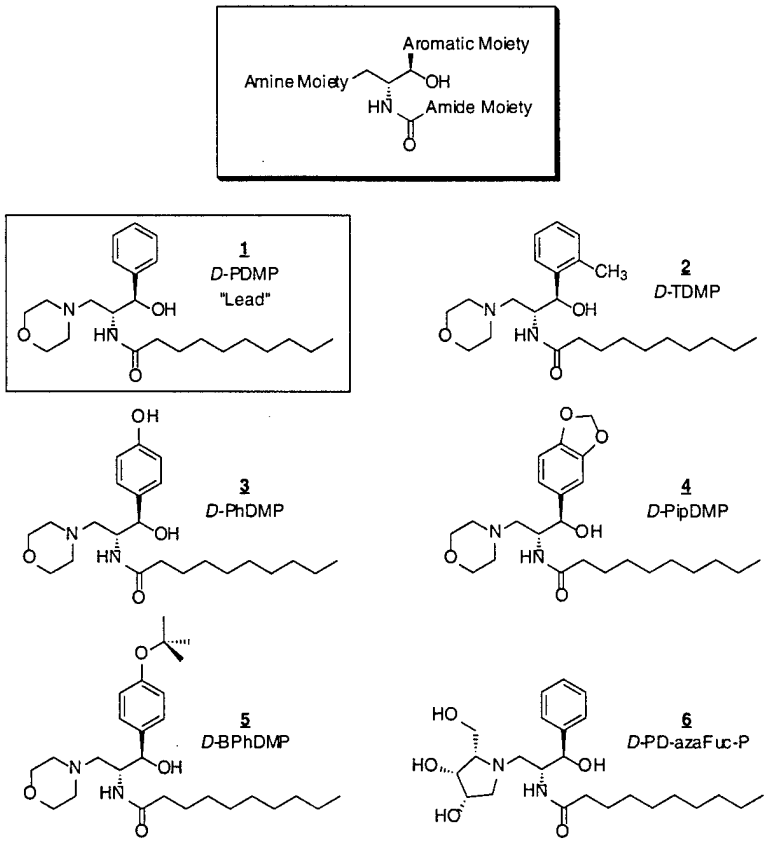


Figure 1. ***PDMP and Analogs.*** Several glucosylceramide synthase inhibitors have been synthesized using the published methodology, based on the lead structure **1**, *D*-PDMP.

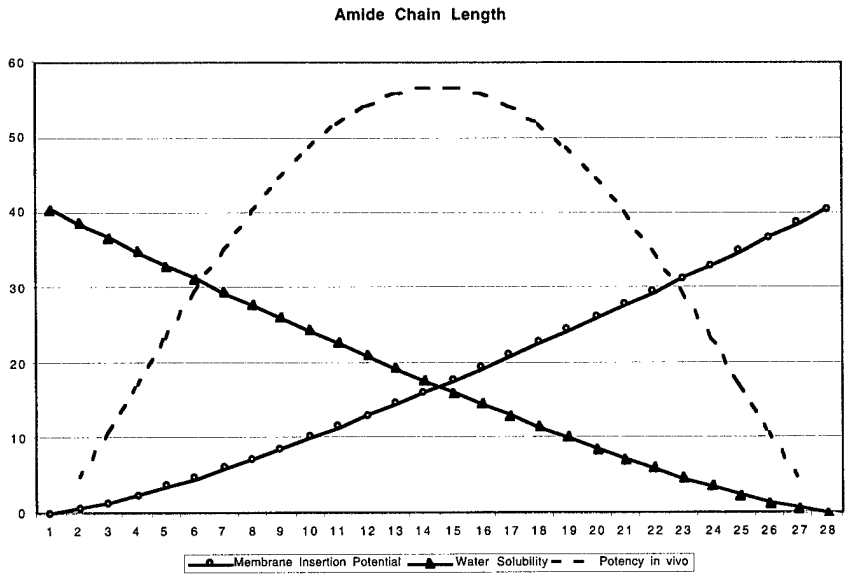


Figure 2. ***Ideal Amide Chain Length.*** Biological activity of a membrane-active drug *in vivo* is a product of the drug's water solubility (▲) and its potential for membrane insertion (O).

The oxygen-substituted aromatic moieties **3**, **4**, and **5**, as well as the *ortho*-methyl derivative **2** are being examined for activity in the antennae explant assay (*vide infra*), and the preparation of compound **6**, bearing the much more sophisticated aza-Fucose amine moiety, will be completed shortly. The choice of the ortho-methyl substituted compound **2** becomes clear in the light of information from the X-ray data obtained in this lab for *D*-PPMP (**Fig. 3**). Substitution of an *ortho*-H with *ortho*-methyl is expected to hinder the rotation of the aromatic moiety, (**Fig. 4**) which may have an impact on the affinity for glucosylceramide synthase.

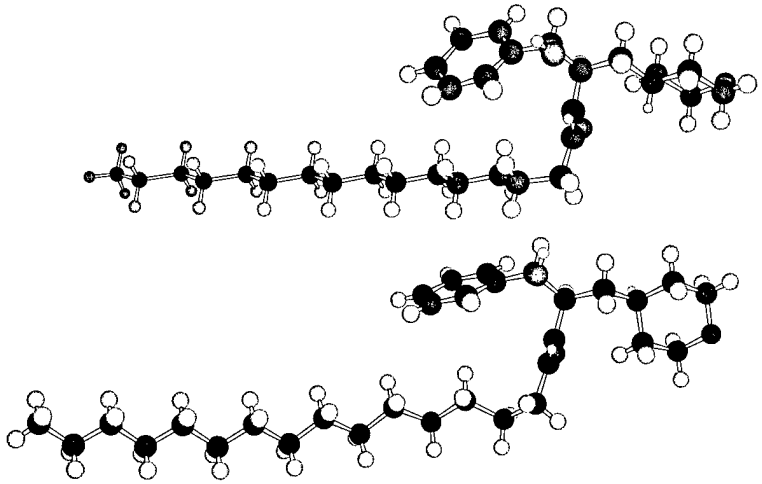


Figure 3. ***X-Ray Structures of L-PPMP•HCl•H_2O.*** Two unique conformations exist in the unit cell. The H_2O molecules and Cl^- counter-ions have been removed for clarity.

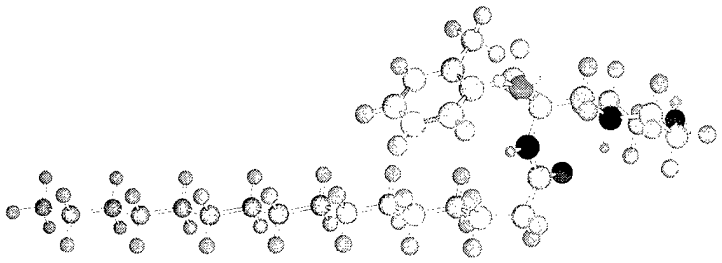


Figure 4. ***Minimized Structure of L-TPMP.*** Presence of the methyl group should hinder rotation of the aromatic moiety. The pharmacological effect on neurite extension is presently being studied.

The structure in **Fig. 4** was obtained by molecular dynamics-driven modification of the X-ray data presented in **Fig. 3**, using the MM2 force field (Cambridgesoft Chem 3D®) on a G4 Macintosh. While this level of treatment is not extremely sophisticated, the molecules are simple enough that more accurate calculations (*e.g.* MacroModel, etc) are probably not justified. Also, the results are clear cut, and in agreement with what would be predicted from first principles. A "dihedral driver" study (**Fig. 5**) clearly indicates that the *o*-methyl group hinders rotation of the aromatic moiety.

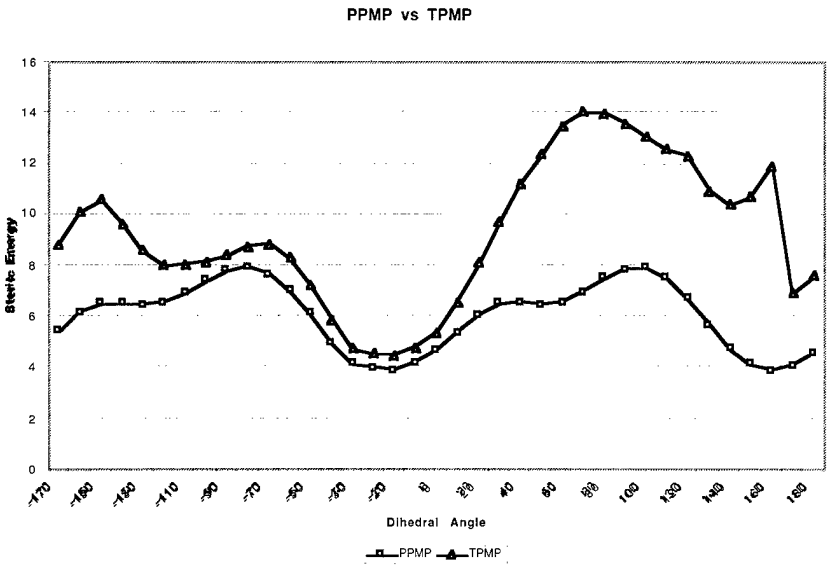


Figure 5. ***Rotation of the Aromatic Moiety.*** Presence of the methyl group breaks the symmetry of the aromatic moiety, destabilizes other rotamers.

This rational approach to drug design clearly complements the combinatorial approach described earlier,[1] which relies on the insect neurite extension assay to identify bioactive conformations. We hope to use the results of the TDMP (TPMP) to guide the formation of libraries biased toward bioactivity.

Bioassay of PDMP Analogs in *M. sexta* Antennae Explants Cultured neuronal explants from the developing antennae of 4th stage pupa phase of development (**Fig. 6**) have been used to assay the effects of the PDMP analogs on neurite extension. Explants (slices) from the developing antennae can be cultured in the presence of PDMP analogs. (**Fig. 7**) Visually, it is clear that increasing levels of a PDMP analog reduces the amount of neuronal outgrowth. It proved difficult, however, to directly *quantify* the effect of a particular concentration of drug on a particular explant by measuring the degree of neurite outgrowth.† This is problematic due to the fact that *a priori* one does not know exactly how many neurons are present in a particular explant, nor exactly which cell type or types are present in a particular explant. Also, it is tedious to measure the linear outgrowth for an explant. It proved to be quite simple however, to determine the concentration of a particular drug that was required to block *all* neurite outgrowth,‡ and thereby obtain a reliable estimate of the efficacy of a particular drug.

Normal Neuronal Development in *M. sexta* Antennae

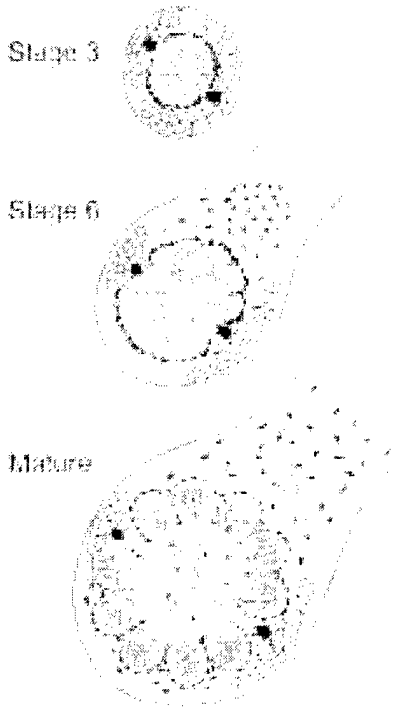


Figure 6. ***Olefactory Neurons Provide Input to Glomeruli During Pupation.*** Developing olefactory axons (green lines) provide a substrate for testing PDMP for inhibition of neurite extension.

† *Imagine trying to measure the length of a strand of spaghetti on a plate. It is much simpler to determine when the plate is empty.*‡

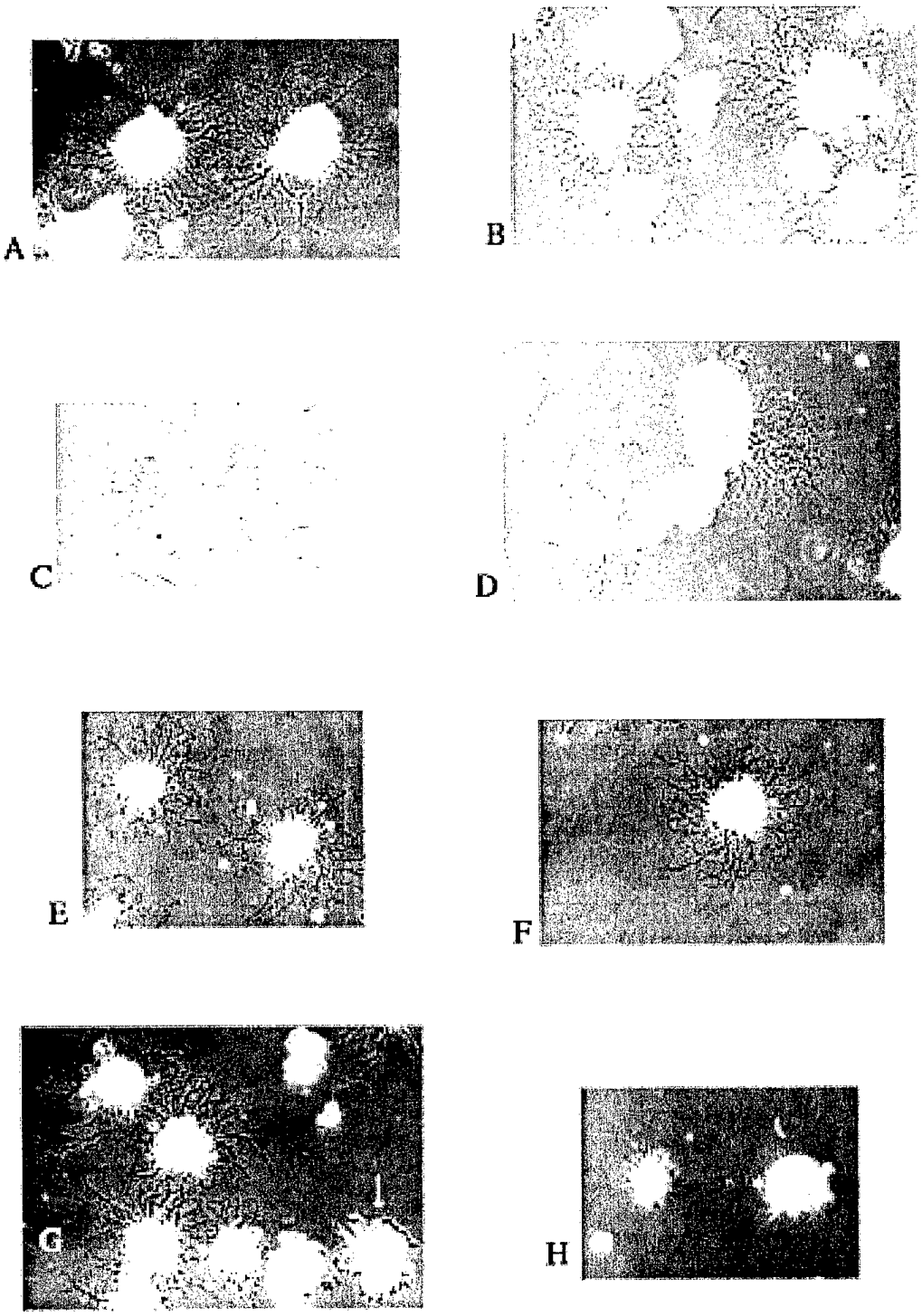


Figure 7. ***Cultured Antennal Nerve Explants.*** Normal axonal outgrowth (neurite extension) from the "sorting region" is shown under low power (10X) in **A** and **B**. Effects of D-*threo*-PPMP at 5 µmol (**C**) and 10 µmol (**D**) concentrations show reduced axonal outgrowth. Effects of L-*threo*-PPPP are shown concentrations of 1 mmol (**E**), 2 µmol (**F**), 5 µmol (**G**), and 10 µmol (**H**). Note that axonal outgrowth is completely inhibited at higher concentrations of L-*threo*-PPPP. No toxicity was noted at these concentrations.

The *Manduca sexta* explants were taken during the 4th stage pupal phase of development. The reasons for using the animal at this particular stage is that it is easily manipulated due to its cocoon stage and its large size. More importantly, it is in the pupal phase that the receptor axons of the olfactory system begin to set up a linked network with the brain of the organism, and should in principle be most susceptible to the effects of the drug. The study was focused upon the effects of the PDMP analogs on the neurite extension of neuronal olfactory cells. These cells are found in the epithelia of the antennae of the *Manduca sexta* during development.

In summary, it was found that the degree of inhibition of neurite extension in the D-series was in the order of D-PPPP > D-PPMP > D-PDMP, which follows the known inhibition of mammalian glucosyl ceramide synthase, which suggests there is a degree of homology between the insect and mammalian forms of this enzyme. Notably, reports that L-PDMP *promotes* neurite extension in mice could *not* be confirmed in *M. sexta*. The L-series had little or no effect on neurite *extension*. Experiments planned for the near future will examine the possibility that the L-series could affect the *direction* of neurite extension. This will be accomplished by placing the explants near target tissues to see if the neurites extend toward the target tissue, or retain a circular extension pattern.

Matrigel Invasion Assays (Human Breast Tumor Cells). In parallel with the insect studies, the effects of several analogs on the invasiveness of human breast cancer cells was observed (Kathy McGovern Lab, U of A Cancer Center). Within the D-series (Glc-Cer inhibition), the degree of inhibition was what was expected (D-PPPP > D-PPMP > D-PDMP). This is logical, based on known inhibition of the mammalian Glc-Cer synthase enzyme. Unexpectedly though, the L-series (enzyme target unknown) proved to be *more effective* against tumor cell invasion.

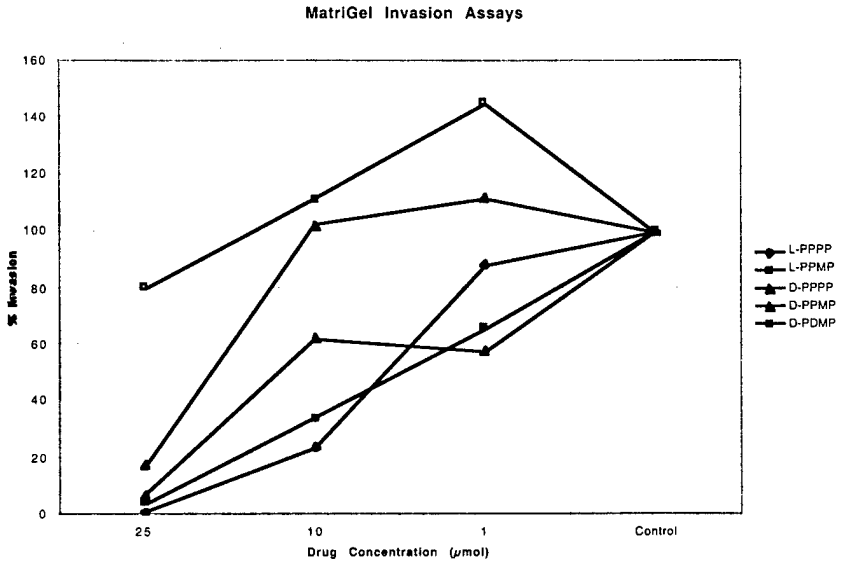


Figure 8. ***Tumor Cell Invasion Assays.*** Invasion of mammalian breast cancer cells through a MatriGel® chamber could be inhibited completely with 25 μmol concentrations of D-PPPP, L-PPMP, and L-PPPP. Near complete inhibition could be achieved with 25 μmol D-PPMP, but minimal effects were observed with D-PDMP.

Chemical Assay of Glycosphingolipid Levels in *M. sexta*. MALDI MS has been the most useful method for screening the large quantity of extracts that have been generated. The analysis is performed on a Bruker Reflex III MALDI-TOF instrument in the MS facility. Based on recent TLC screenings of extracts using our microwave extraction protocol we observed that the larva that had the guts removed had different TLC profiles than intact larva (see section 3 below). We suspect that in the course of the extraction we were coextracting compounds from the larva's food that remained in the gut. The stomach contents, when freeze-dried, constituted approximately 25% of the mass of the tissue. Subsequently, we dissected 5 larva to remove the guts prior to freeze-drying and subjected the remaining tissue to microwave extraction using

2:1 $CHCl_3/CH_3OH$. The extraction solvent was removed and the solid was redissolved in 20 ml 2:1 CHC_3/CH_3OH to give a nominal concentration of 25 mg/ml.

The samples were subjected to both MALDI and high resolution MALDI analysis. In the high-resolution experiments, the mass range from 300—2500 m/z was calibrated with CCA matrix dimer, Angotensin II, Bradykinin, LHRH, Bombesin, alpha-MSH and ACTH (18-39).

Putative GSL structures have been proposed, based on the MS data. (**Fig. 9**) In addition, there are m/z values that could not immediately be assigned a molecular formula that would be consistent with GSLs. The samples will also be analyzed by MALDI *Post Source Decay* (PSD) and *Linked Scanning* on the magnetic sector instrument. MALDI PSD has been run on previous extracts with limited success. Since this was the first time we have attempted this experiment, we need to spend more time optimizing parameters for this class of compounds.

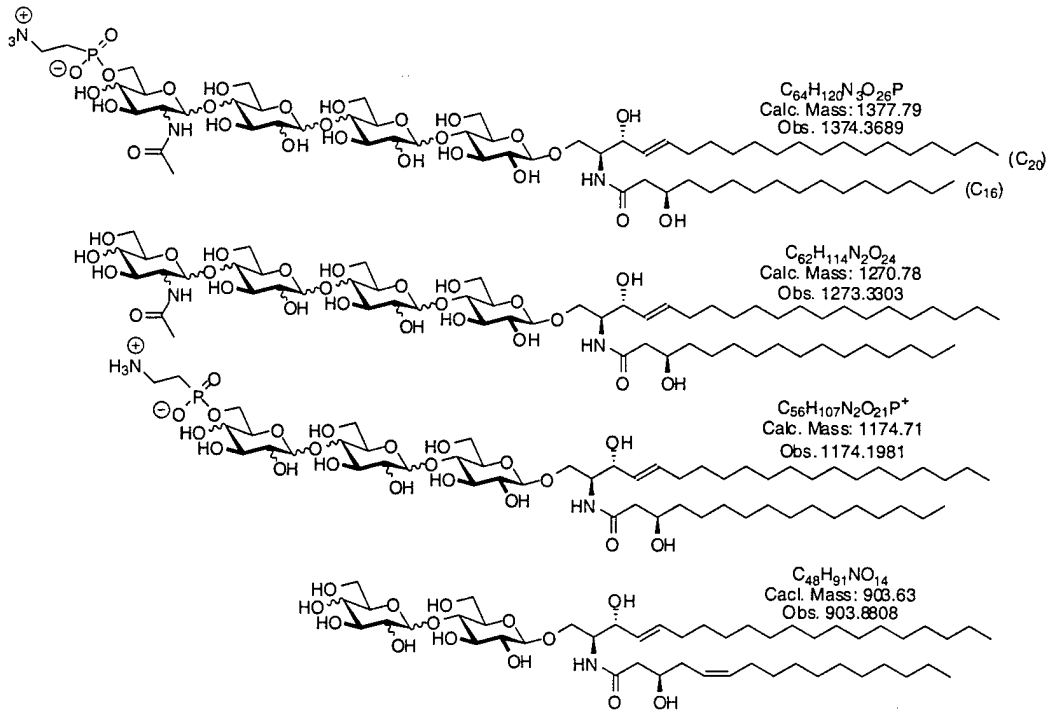


Figure 9. ***Putative GSL's Structures Observed in MALDI Spectra.*** Precise information on the identity of the saccharides cannot be obtained from MALDI. The sugars depicted should be regarded as "hexoses."

Electrospray Analysis. Using the Extrel 4000 ESI-MS in the Wysocki lab, we have experienced difficulty electrospraying the extracts using the current source. In an effort to get a solvent system more compatible with electrospray conditions, an attempt was made to

derivatize the GSL. Using a standard GSL from Avanti Polar Lipids, the GSL was converted to the imidate salt using the protocol published by Weintraub.[2] In principle, this procedure fixes a charge on the amide nitrogen, allowing dissolution of the sample in 100% methanol in order to efficiently electrospray the solution. However, we did not observed molecular ions when the derivatized GSL was electrosprayed. This approach may be useful for fragmentation approaches, however.

Thin Layer Chromatography As mentioned above, a recent screening of dissected larva suggested that we have been coextracting compounds from the larva's wheat germ food. Based on the report of Sullards,[3] wheat germ is known to contain several GSLs. Based on the TLC profile, we anticipate using only dissected larva in the future to ensure that the GSLs isolated are from the larva and not from the food.

Microwave Enhanced Extraction *of Manduca sexta.* In an effort to reduce solvent consumption and speed extractions we developed a microwave extraction protocol involving first, the washing of the freeze dried tissue in acetone to remove the lipids followed by multiple 2:1 $CHCl_3/CH_3OH$ extractions. In order to optimize the method, several extractions were run at different times to evaluate the quantity of material extracted. In summary, it was found that this approach dramatically decreased the time required for a Folch extraction (10 min *vs* 2 h), and increased the efficiency of the extraction process as well.

High Pressure Liquid Chromatography. A state-of-the-art HPLC system (Varian Star System) was put in place in late July of this year, and should permit the isolation of individual GSLs. This should facilitate the precise characterization of the glycosides by NMR, and facilitate identification by MS methods. In addition, it is anticipated that pure samples of the GSLs will permit the generation of fluorescent antibody reagents for the identification and quantification of individual GSLs in tissue samples. Ultimately, this will be important to determine cellular and sub-cellular location of GSLs in *M. sexta.*

Key Research Accomplishments

- Demonstrated a clear correlation between activity in the insect neurite extension assay (arthropod system) and the tumor cell invasion assay (mammalian system) for the D-series of PDMP compounds.
- Demonstrated a *lack* of activity in the insect neurite extension assay for the L-series of PDMP compounds.
- Demonstrated strong activity in the tumor cell invasion assay for the L-series of PDMP compounds.
- Demonstrated the utility of removing the gut of the *manduca* prior to extraction to prevent extraction of GSL from *manduca* foodstuffs.
- Developed improved extraction procedures for GSLs using a commercial microwave to speed the extraction process and enhance the efficiency of the extraction.
- Have identified candidate peaks in the MS for further study.
- Have explored the use of TLC/MS as an alternative to HPLC, and defined its limitations.
- Have set up a state-of-the-art HPLC system for isolation of GSLs.
- Have produced a number of new PDMP analogs for study.

Reportable Outcomes

1) D-*threo*-PDMP and Structural Analogs Alter GSL Expression in Manduca sexta and Reduce Neurite Extension in Explants.
Robin Polt, Lynne Oland, Chris Biland, James B. Glick, Bennett Novak, Jacob Slavish, Will H. Taylor, Kathy McGovern and John Hildebrand
National Academy of Sciences' Colloquium: "Molecular Kinesis in Cellular Function and Plasticity." 7-9 Dec. 2000, Beckman Center, Irvine, CA.

2) Lipo α-Amino-β-Hydroxy Acids and O-Linked Glycosides: Building Blocks for Ceramyl and Glycosphingoyl Peptides
Michael M. Palian and Robin Polt
Journal of Organic Chemistry (in press)

3) Isolation and Identification of Glycosphingolipids from the *Manduca sexta*
William Taylor, Thesis, Department of Biochemistry, University of Arizona (2001)

Conclusions

An important intellectual advance in our understanding of GSL function has been made, in that credible molecular functions for GSL "rafts" (formerly GSL "patches") have now been assigned. Essentially, the GSLs may function as recognition elements that allow sphingomyelin-cholesterol membrane units to deliver proteins and receptors to particular membrane surfaces in a cell.[4] (**Fig. 10**) Thus, it seems clear that if D-PDMP and analogs disrupt the biosynthesis of GSLs, then delivery of important receptors will be disrupted, resulting in changes in cell behavior (*e.g.* neurite extension). This is important in an intellectual sense, because it now provides a complete rationale for the exploration of D-PDMP and other drugs that can alter GSL expression.

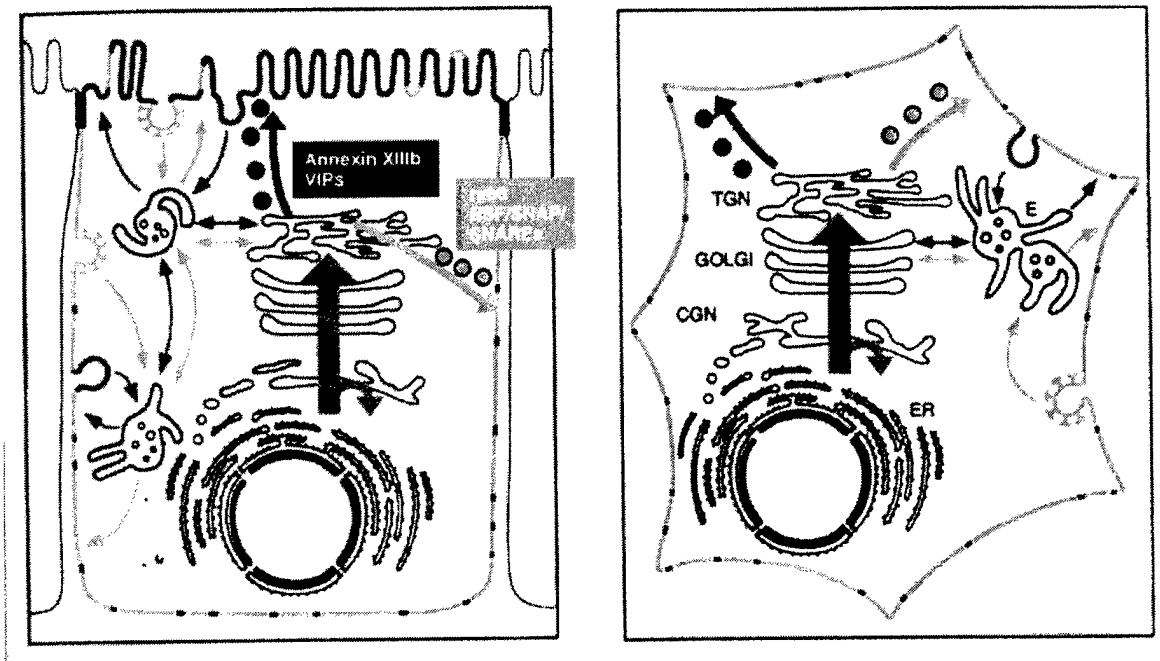


Figure 10. ***GSLs Play Important an Important Role in Rafts.*** Rafts have been demonstrated to deliver receptors to particular surfaces in skin cells (left) and in fibroblasts (right).[4]

What about the effects of L-PDMP? While this requires some speculation, it seems clear now that sphingosine phosphate plays an important role in the activation of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) to provide for cell motility.[5] (**Fig. 11**) While the D-series of PDMP compounds clearly act on glucosylceramide synthase, the L-compound may act directly on sphingosine-processing enzymes (*i.e.* sphingosine kinase).

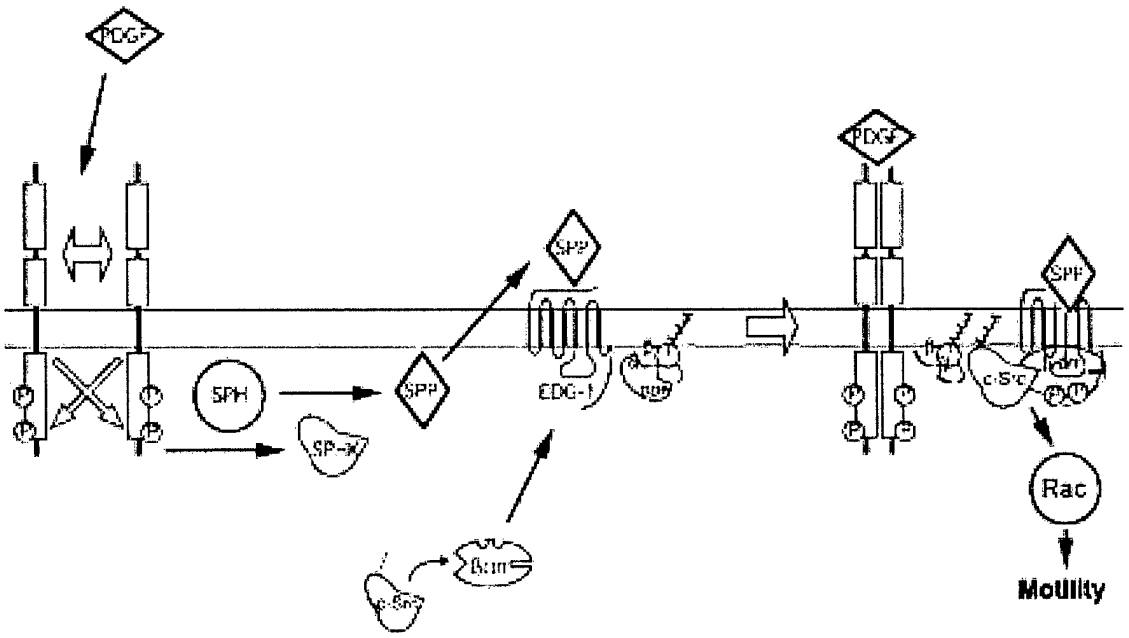


Figure 11. ***Sphingosine Phosphate Plays a Key Role in Cell Motility.***[5] Both EDG-1 and PDGF must be activated to induce cell motility. One possible function for the L-PDMP compounds may be to block the action of sphingosine kinase, either directly or by preventing the formation of free sphingosine from sphingomyelin.

We are now in a position to move rapidly toward completion of this project. Significant technical, equipment and personnel problems have been overcome. Dr. Bennett Novak has moved on to a position in industry (DuPont, Midland, TX), and a new post-doctoral appointee with experience in the isolation of water soluble natural products (and excellent graduate training) should fill his position shortly. Dr. D.T.U. Abeytunga, who has a Lecturer position at the University of Columbo, Sri Lanka, will fill this position by 1 September.

References

[1] Glycosyltransferase Inhibitors: Synthesis of D-threo-PDMP, L-threo-PDMP and Other Brain Glucosylceramide Synthase Inhibitors from D- or L-Serine. Mitchell, S.A.; Oates, B.D.; Razavi, H.; Polt, R. *J. Org. Chem.* **63**, 8837—8842 (1998)

[2] Weintraub, *et al. J. Org. Chem.* **33**, 1679 (1967)

[3] Sullards, *et al. J. Mass Spectrum.* **35**, 347-353 (2000)

[4] K. Simons & Elina Ikonen *Nature* **387**, 569 (1997)

[5] a) S. Spiegel & S. Milstein *Biochim Biophys Acta* **1484**, 107-16 (2000), b) John P. Hobson, et al. Science 291, 1800-1803 (2001)

Isolation and Identification of Glycosphingolipids from the *Manduca Sexta*

BY
William Taylor

A Thesis Submitted to the

DEPARTMENT OF BIOCHEMISTRY

In Partial Fulfillment of the Requirement
for the Degree of

Biochemistry, Bachelor of Arts

In the Undergraduate College of

THE UNIVERSITY OF ARIZONA

P.I.: Robin Polt, Ph. D

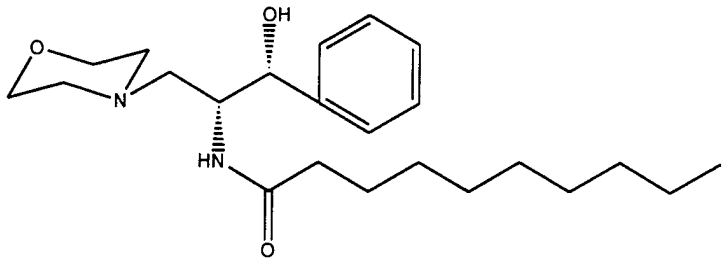
James G. Glick

CONTENTS

I. Introduction

Purpose and Goal

The study of this research has two main goals: **(1)** To develop a method that will isolate and identify the major glycosphingolipid components of the Manduca Sexta and **(2)** to perturb the pathway of glycosphingolipid biosynthesis via the usage of ceramide analogs, thereby revealing their role in neurite growth. Examples of these ceramide analogs are D/L-*threo*-PDMP (fig. 1), D/L-*threo*-PPPP, and D/L-*threo*-PPMP.



PDMP

Fig. 1: The chemical structure of PDMP

The reason that the *Manduca Sexta* is used as a standard system for GSL research is quite simple. The Manduca Sexta has become a standard model system in the research field of neural development, motor control, visual-motor behavior, hormonal control of development, and olfaction research. The *Manduca Sexta* is an invertebrate with a GSL metabolism system that is comparative to that of human beings while, at the same time it is much less complex. This meaning that the lack of salvage and repair systems in the *Manduca Sexta* will allow for the results of blocking pathways to be more readily identifiable. Also the biological aspects of this creature are very well understood. The *Manduca Sexta* has a short maturation and life span, with a life cycle that spans over a

time period of approximately eight weeks. This makes it desirable for the sake of research because it is favorably short yet it is slow enough that distinct stages of development can be isolated and experimented with. Also, due to the distinct phases of maturation that it passes through and the inexpensive cost of food for this creature support its desirability for being the research model. Since they are reasonably easy to reproduce, large numbers can be utilized for the isolation procedures to obtain glycosphingolipids. In parallel, we have begun experiments using ceramide analogs to perturb the GSL pathways of the *Manduca Sexta*. The Manduca Sexta is desirable here because much is already known about its nervous system. These effects have been observed and recorded by growing neuronal tissue explants in the presence of varying levels of these ceramide analogs. The effects are apparent: an increased level in the ceramide analog concentration leads to a decrease in neural processes growth.

Fig 2: The life cycle of the *Manduca Sexta*

Illustrated above is the life development cycle of the Manduca Sexta. For the glycosphingolipid isolation research, the 5^{th} instar stage larva was used (fourth step clockwise from the moth). For the glycosphingolipid perturbation research, the 4^{th} stage pupa was used (second step clockwise from the moth)

History and Structure

Glycosphingolipids are cell membrane surface components that are found on the outer membrane surfaces of all multicellular organisms (eukaryotes). Glycosphingolipids were first described during the second half of the nineteenth century. The structural identification and investigation of glycosphingolipids was accelerated by access to autopsy material obtained from patients who had been diagnosed with rare metabolic diseases. In these rare diseases glycosphingolipids accumulate over the time period of their storage due to the cell's inability to catabolize them. This fatal storage of glycosphingolipids is due to mutations that result in a deficiency of the proteins needed to carry out their lysosomal degradation. Examples of these lysosomal storage diseases of glycosphingolipids are Tay-Sachs disease and Sandhoffs disease (**9**). These diseases lead to the degeneration of the nervous system in those that are affected by it.

These compounds play important roles in biological development, maintenance, and the nervous system repair mechanisms of humans and other organisms. An understanding of their structure, function and regulation is necessary in alluding to the role they play in cells. Glycosphingolipids can basically be generally stated as being carbohydrate-bearing sphingosines or ceramides. Glycolipids are made of a hydrophobic ceramide (N-acylsphingosine) moiety and one or more sugars linked by a glycosidic bond to the terminal primary hydroxyl group of sphingosine (**4**).

Glycolipids, in general, are heterogeneous in both the oligosaccharide and the ceramide portions, but they are usually characterized based on the composition of their carbohydrate structures. The major forms of these sugars that are found in animals,

including humans, are glucose (Glc), galactose (Gal), fucose (Fuc), and N-acetylgalactosamine (GlcNAc) (**16**). There are basically four main categories that the glycolipids are divided into: cerebrosides, gangliosides, arthrosides and sulfatides. Gangliosides are glycolipids that contain one or more sialic acid moieties attached to a neutral sugar by an α-ketosidic bond. Sulfatides are glycolipids in which the carbohydrate moiety contains a sulfate group. Arthrosides are those glycolipids that are found on most insects and do not contain a sialic acid functional group. Cerebrosides compose a major part of the structure of two vitally important cell membranes, which control the flow of water, and nutrients into the plant cell and sequester waste products.

The majority of glycolipids are derived from glucosylceramide or galactosylceramide by sequential addition of monosaccharides to these core structures. The fatty acid components of glycolipids are usually more complex than the ones found in core structures. These fatty acids range in their complexity from C_{14} to C_{24} in chain length. The glycolipids mostly found in mammalian cells generally contain fatty acids of short chain length, C_{16} to C_{18}. More than 400 species of glycosphingolipids possessing different sugar structures have been reported, however, only seven of these are mainly found in vertebrates (**16**).

Arthrosides vs. Gangliosides

In arthropods the structures of glycosphingolipids are simpler than those found in mammals. Most glycosphingolipids have by categorized by identification of their core structure. Arthropods contain arthrosides, which differ from gangliosides because they contain no sialic acid or fucose. The purpose of the fucose and sialic acid in higher

organisms is to modify the terminal carbohydrate residues to influence the cellular immune system. This occurs in the gangliosides (**17**). Not much is known about arthrosides simply because they have not been extensively studied. What is known is that in each species only a few types of glycosphingolipids will be present. This glycosphingolipid expression will be similar between species that are closely related and as the species gap increases so does the difference in GSL cell expression. An example of this would be the similarity between the glycosphingolipid expression found on pig and human cells. Xenotransplantation between pig and human organs is made possible by this similarity.

Some common examples of gangliosides are the antigens that determine blood type, the Lewis antigen system responsible for recruitment of leukocytes to sites of injury, and in cancer, glycosphingolipids act as tumor cell markers classifying tumor cells as benign/malignant, invasive/noninvasive, etc. These gangliosides also play critical roles in cell-cell communication, cell adhesion, proliferation, axonal path finding, neuronal growth and repair, cell transformation, tumor progression, and immune response (**7**). Arthrosides are believed to perform many of the same mammalian cellular-molecular functions that gangliosides perform. The advantage of arthrosides is that they are found in insects and can be studied to gain insight to the function of gangliosides. The insect system is very advantageous for use due to the short time period of development and the ease of reproduction in high numbers. It is thought that arthrosides could have similar structural composition of gangliosides with the presence of glucuronic

acid or phosphoethanolamine in the overall structure of the arthrosides compound. These acidic moieties would mimic the sialic acid groups that are found in gangliosides.

Glycosphingolipid Cell Distribution and Functionality

A carbohydrate layer covers the surface of eukaryotic cells. This layer has been termed the glycocalix and it consists of glycoproteins, glycolipids, and glucosaminoglycans. The ceramide portion has the essential function of anchoring the lipid-bound carbohydrates in the membrane of the eukaryotic cells. Also, the ceramide portion is thought to be involved as an influential factor in the localization and functions of glycosphingolipids on the plasma membrane.

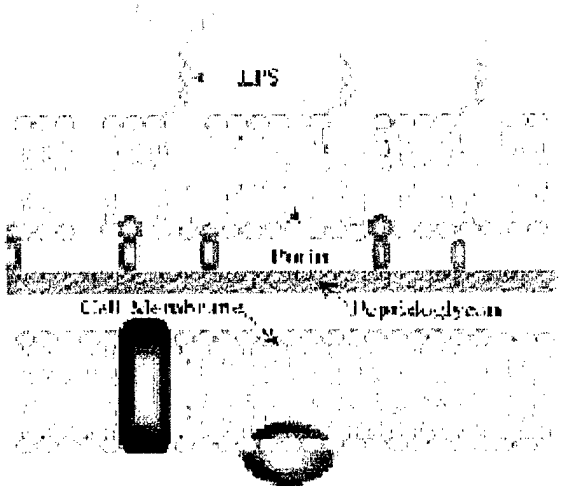


Fig 3: Membrane localization of GSLs (shown here as lipopolysaccharides: LPSs) (7).

This could be correlated with the direct interactions GSLs have with cholesterol, phospholipids, and the transmembrane domains of receptor proteins. Free ceramide could also possibly play a role in mediating intracellular signal transduction. The lipids can be classified into a few series that are characteristic of species related in their evolutionary history. These glycosphingolipids form certain functional patterns on the

cell membrane surface that can change according to the differentiation stage of the cell or with viral and oncogenic transformations (**9**).

Glycosphingolipids show a non-uniform distribution in the plasma membrane. They seem to be restricted to exoplasmic leaflets of the plasma membrane and the lumenal side of organelles where they have the tendency to cluster in patches. It should be noted that the possibility of free glycosphingolipids being present in the cytosol has not been ruled out. There are certain invaginated areas on the cell's exoplasmic membrane that are known as caveolae. These caveolae are highly susceptive to endocytosis. Also, these caveolae contain high concentrations of glycosphingolipids and signal transducing proteins. The presence of the glycosphingolipids in the same area as these proteins could support the idea that these glycosphingolipids are in fact involved in signal transduction. Another interesting characteristic about glycosphingolipids is that they have the ability to move laterally around on the membrane. Glycosphingolipids package their information as chemical coding within the carbohydrate moieties. These carbohydrates range from monosaccharides to enormous oligomeric structures that have been found in macromolecular proportions. Glycosphingolipids have the tendency to cluster together on the exoplasmic membrane. These cluster areas also contain cholesterol and a relatively low concentration of phospholipids. With the combination of their clustering characteristic and their ability to move laterally, glycosphingolipid-rich 'rafts' (fig. 4) are formed (**9**).

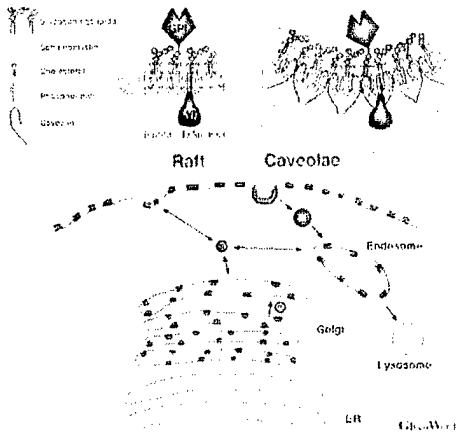


Fig 4: Cell membrane location and metabolic cycle of GSLs (7).

It has been observed that there are signal transducers on the exoplasmic face of the rafts and there are *src* family kinases on the cytosolic face. This is yet more indication of their involvement in transmembrane signal transduction (**5**). These rafts are often trapped in the caveolae causing their receptor molecules to be clustered more densely. Also, during development these glycosphingolipids allow the cells to recognize one another. This could either promote or inhibit cells from moving into adjacency with them. The thing that is interesting about glycosphingolipids is that they are composed of a variety of structures that act together to display one message. Due to this "ensemble of structures", the production units for glycosphingolipids are found dispersed throughout the entire cell.

Biosynthesis

As mentioned, there are certain glycosphingolipid patterns that are characteristic for a cell type in certain stages of cellular development that are found on the cell surface. This indicates that there are coordinated and interrelated processes involved in the biosynthesis, degradation, and intracellular transport of glycosphingolipids. Biological membranes are composed of lipid bilayers that consist of cytosolic and anticytosolic layers that can simultaneously be of different composition. Glycosphingolipid biosynthesis and degradation occurs in different cellular organelles. The precursors to

glycosphingolipids are found in intracellular membranes that are associated with the plasma membrane via membrane fusion processes. The enzymes that are involved in glycosphingolipid biosynthesis are membrane-bound proteins. The de novo biosynthesis of glycosphingolipids occurs in the same compartments as glycoproteins (**9**). This biosynthesis is coupled to the intracellular vesicular transport of the growing lipid molecule that leads to the plasma membrane through the cisternae of the Golgi apparatus. Biosynthesis begins with the formation of ceramide on the membranes of the endoplasmic reticulum. The steps of this ceramide synthesis are as such: the amino acid L-serine undergoes condensation with palmitoyl-CoA to yield 3-ketosphinganine; 3-ketosphinganine is reduced to D-erythro-sphinganine; D-erythro-sphinganine condenses with an activated fatty acid to finally yield ceramide. This ceramide will then undergo glycosylation to yield glycosphingolipids (**13**). The first glycosylation occurs by - glycosidically linking a sugar to the 1-position of the ceramide. This and other carbohydrate additions are catalyzed by a group of enzymes known as glycosyltransferases. One common reaction that occurs is the galactosylation of ceramide to yield galactosyl ceramide. This compound, along with sulfatides, is found in high concentrations in the axons of neuronal cells. However, the biosynthesis of most vertebrate glycosphingolipids, as in humans, begins with glucosylation of ceramide to give glucosylceramide. These reactions work by the transfer of UDP-activated sugars to the ceramide. By inhibiting the enzyme, glucosyl transferase, that catalyzes this reaction the importance and vital role of glycosphingolipids in morphogenesis and embryogenesis

can be demonstrated. One interesting note about ceramide is that it is also the precursor to sphingomyelin. Sphingomyelin is structural component of the plasma membrane.

The transport system used during glycosphingolipid biosynthesis is quite important and interesting. The first four steps, as previously mentioned, produce ceramide and the reactions occur via membrane bound enzymes at the cytosolic dace of the endoplasmic reticulum. The ceramide is then transported to the cytosolic face of the Golgi apparatus, also known as the pre-Golgi compartment. Here the formation of glucosylceramide occurs. This is then transported to the luminal site of the Golgi apparatus where the introduction of the next sugar residue occurs which results in the formation of lactosylceramide. A flippase enzyme performs this transport from the cytosolic area to the luminal. The biosynthesis of higher gangliosides also occurs on the luminal side of the Golgi apparatus (**13**).

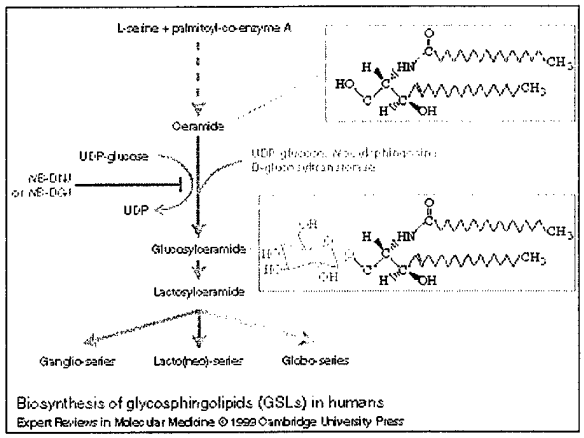


Fig 5: Biosynthetic pathway of glycosphingolpids (7).

Catabolism

When it is time for the cells to change their extracellular chemical expression, the alteration of glycosphingolipids must take place. The balance of synthesis and

degradation is completely regulated in the cell. After the glycosphingolipids recycle between the plasma membrane and intracellular organs, the glycosphingolipids are endocytotically transported to the lysosomes. The degradation of sphingolipids takes place in the lysosomes of the cell. Lysosomes membrane-bound organelles found in the cytoplasm of most cells and contain various hydrolytic enzymes that function in intracellular digestion. Here the glycosphingolipids are all hydrolyzed sequentially from the non-reducing end by exo-type glycosylhydrolases. These hydrolases that carry out the degradation of the glycosphingolipids are dissolved in lysosol. Ceramide is formed from the lower glycosylated sphingolipids. It is degraded to sphingosine and a long chain fatty acid. The degradation of sphingolipids occurs selectively in the lysosomes without compromising their integrity. This lysosomal degradation begins at the surface of the intralysosomal vesicles. The vesicles are the caveolae that are previously mentioned. They have high glycosphingolipid content. These areas will invaginate into a vesicle that is then attacked by a lysosome and the innercontainings are degraded. Another means of glycosphingolipid degradation is to pull it out of the membrane, as it is. The glycosphingolipid is pulled out of the membrane by an activated protein, a liftase, which allows it to be mobile in a water-soluble manner. Now the degrading enzyme comes in and attacks the sphingolipid (**9**). The degradation process is a sequential one in which the terminal sugars are cleaved away one at a time. All of these hydrolysis reactions require an activator protein that acts as a detergent to isolate the glycosphingolipids that it is specific for. It is when these hydrolases are not functioning correctly that the

synthesis/degradation balance is disturbed and the lysosomal storage diseases are initiated.

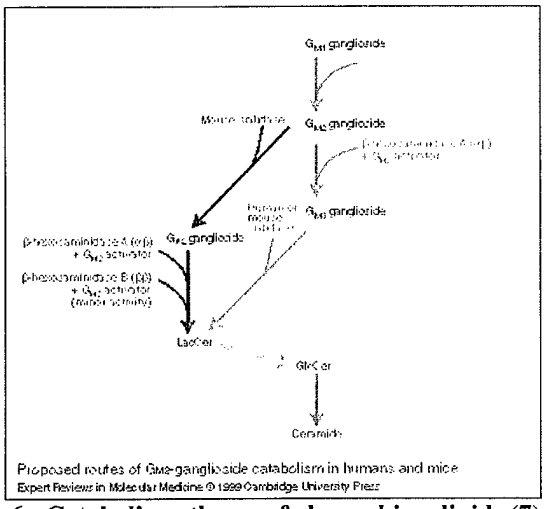


Fig 6: Catabolic pathway of glycosphingolipids (7).

Inhibition of glycosphingolipid function

Using ceramide analogs to inhibit glycosphingolipid biosynthesis has presented the hopes of progress towards discovering and understanding their cellular role. The steps of formation for glycosphingolipids is that first the membrane anchor is formed. The next step is the addition of the carbohydrate groups by way of the glycosyltransferases. It is during the early steps of glycosylation that inhibition is possible. It is possible to inhibit the biosynthesis of ceramide altogether. This, however, would be pointless due to the resulting side effects of such an inhibition: a depletion of glycosphingolipids in the plasma membrane, a reduction in sphingomyelin biosynthesis, and an increase in the concentration of the metabolites located upstream of the inhibited step (**1**). The most appropriate inhibitors for this research are those that affect GlcCer

biosynthesis. The most studied inhibitor of the GlcCer synthase in D-*threo*-PDMP (D-*threo*-(1R,2R)-1-phenyl-2-decanoylamino-3-morpholino-1-propanol).

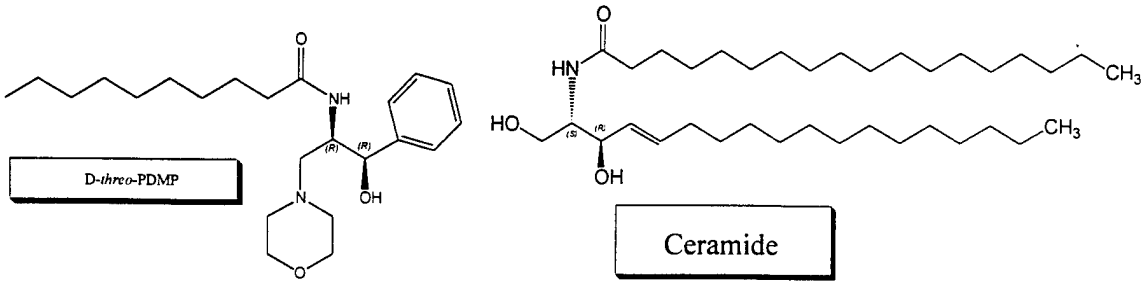


Fig 7: A visual comparison between the structures of PDMP and ceramide.

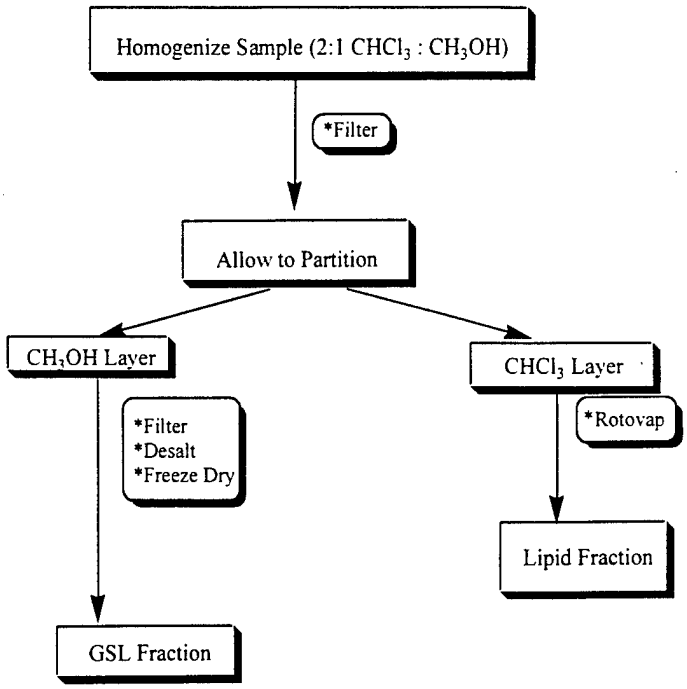
It is quite obvious upon comparison that PDMP has a similar structure to ceramide. D-*threo*-PDMP is able to inhibit the formation of glycosyl-ceramide in concentrations of 2.5-10 M. D-*threo*-PDMP displays a mixed inhibitor profile relative to ceramide and is noncompetitive for the glycosyl donor. The determined K*i* is 0.7 M. The effects of D-*threo*-PDMP on the cell are: inhibition of cell growth and inhibition of neural processes growth and blockage of sphingolipid membrane transport from the Golgi apparatus to the ER (**2**).

II. Methods

Glycosphingolipid Isolation and Identification

The primary goal of the research explained in this paper is the isolation and identification of glycosphingolipids from the Manduca Sexta. The methods used for the isolation of the glycosphingolipids begin with the liquid extraction of the glycosphingolipids from the tissue. This is shown in the protocol.

Folch Isolation Protocol for Glycosphingolipids



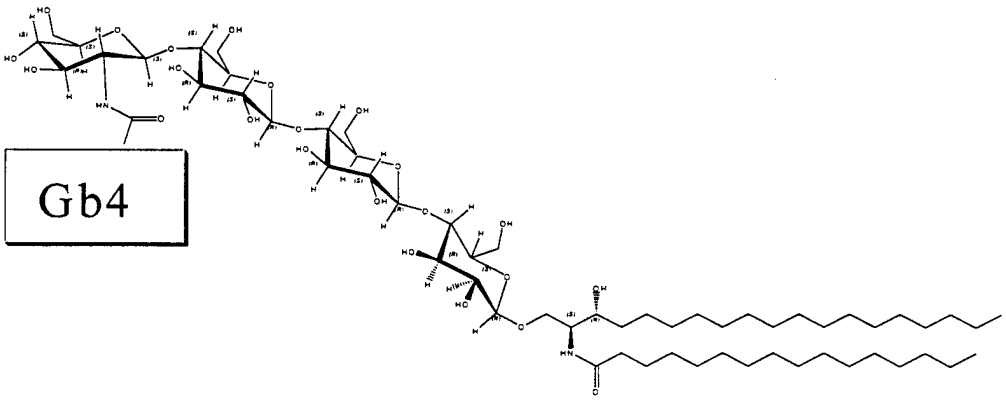
‡Folch et. al. J. Biol. Chem. **226,** 497 (1957)

Fig 7: Visual illustration of the isolation protocol used.

The next step that is carried out is the lyophilization of the aqueous, glycosphingolipid-rich liquid. This then leads into and prepares for the process of column chromatography using Sephadex to separate the neutral glycosphingolipids from the acidic glycosphingolipids. These fractions are then run through mass spectrometry analysis (FAB, MALDI, & CID). This is accompanied by gas chromatography, which attempts to determine the location of the points of unsaturation. The combined efforts of these methods will lead to the identification of an isolated GSL. The first step of the process was to obtain the Manduca Sexta from the Tolbert/ Oland lab of the University of Arizona Neuroscience Department. The average number of organisms that were obtained

was ten 5th-instar Manduca Sexta. This gives an approximate sample mass of 100 grams. These organisms were then placed in the freezer overnight to ensure the most humane treatment possible. The organisms were then placed in a standard kitchen blender and were ground into a liquid using the "Frappe" option on the blender. The resulting liquid mixture was that of a green and black sludge. This resulting liquid was then placed in a 500mL round bottom flask and a 2:1 mixture of chloroform: methanol was added. The mixture was stirred rigorously for ten to fifteen minutes. This resulting mixture was then immediately filtered (using #1 Whatman filter paper--24cm.) into a 500mL separatory funnel. The solid material that was trapped by the filter was again placed in a round bottom flask and mixed with 2:1 chloroform: methanol. The resulting mixture was again filtered into the separatory funnel. The contents of the separatory funnel are allowed to partition completely in the two separate chloroform and methanol phases. The methanol layer partitioned into the top layer of the flask and had a tan coloration. The chloroform layer partitioned to the bottom of the flask and had a yellow-green coloration. Both the layers were collected separately and were rotovapped down, this meaning that methanol and chloroform were removed via evaporation and a vacuumed atmosphere. The remains in both flasks were an oily liquid. The MeOH layer is suspected to contain the glycosphingolipids that are attempting to be isolated. The chloroform layer is suspected of containing fatty acids and other hydrophobic compounds. The methanol layer is then taken and lyophilized. This results in the yield of a brown solid that is scraped into a powder and collected. This brown methanol layer extract is then placed on a Sephadex column. The column was prepared wet with a 10:

10: 1 CHCl3: CH3OH: H2O solution. Then 0.6g of the compound was dissolved in 1: 1: 1 CHCl3: CH3OH: H20 to make a light brown solution (**15**). The column was run and two separate eluations were collected. The first solution that eluted off the column was a golden brown residue that contained the neutral and zwitterionic glycosphingolipids. The second solution that eluted off was clear in color and contained the acidic glycosphingolipids. The acidic glycosphingolipid content was confirmed via the use of thin layer chromatography and mass spectrometry (**15**). In order to help ensure that this was in fact a glycosphingolipid compound that had been isolated, mass spectrometry and thin layer chromatography was run on a standard glycosphingolipid, Gb4 (purchased from Matreya).



For the TLC method it was found that the glycosphingolipid standard, Gb4, had an R_f value of 0.52 +/- 0.06. This would hypothetically indicate that if an unknown sample were to have a similar Rf value then there was a greater possibility that it is a glycosphingolipid of some sort. When running thin layer chromatography on any of these glycolipid-containing compounds an Orcinol-based stain was used. This developing reagent consisted of 0.2g: 96mL: 4mL napthelenediol: ethanol: sulfuric acid.

The solvent used for TLC here was a 75: 5: 25 NPA: NH3OH: H2O mixture (**15**). Mass spectrometry interpretations were done mainly using the MALDI and ESI techniques. One can interpret a glycosphingolipid using these methods by looking for sequential fragmentation of the GSL and viewing the masses of each fragment.

Perturbation of the Glycosphingolipid Pathways

In parallel, we have begun experiments using the ceramide analogs to perturb the GSL pathways of the *Manduca Sexta.* This particular study had three specific goals:

1) To develop a convenient, insect based assay system for the development of metastasis inhibitors.

2) To discover lead compounds for the inhibition of metastasis.

3) To unravel the structure activity relationship of glycosphingolipids by using the simple insects, which lack the immunological complications of vertebrates.

These effects have been observed and recorded by growing neuronal tissue explants of *Manduca Sexta* in the presence of varying levels of these ceramide analogs. The *Manduca Sexta* that were used here were in the 4^{th} stage pupa phase of development. The reason for using the animal at this particular stage is that it is easily manipulated due to its cocoon stage and is quite large. Also, the developing antennae are easily manipulated to yield the isolated olfactory tissue. The study here will be focused upon the effects of the ceramide analogs on the cell growth of neuronal olfactory cells. These cells are found on the epithelia of the antennae of the Manduca Sexta. It is in the pupal phase that the receptor axons of the olfactory system begin to set up a linked network with the brain of the organism.

This perturbation process begins with the extraction of the antennae from the Manduca Sexta. The protocol used is as follows:

1) Selection of the necessary number of fourth stage pupa. This is done by observing the development of their legs via the use of a light microscope.
2) Place the selected organisms in ice for ~30 minutes. This will humanely prepare for dissection and also entirely sequester their responsive movements.
3) Take the sequestered organisms and remove their antennae using dissection methods that are as sterile as possible. Filet the antenna and remove as the vein and as much scalar tissue as possible. Place the antenna in a black-capped plastic tube.
4) Obtain 10xHBSS, Culture Chow, IL-15 at room temperature.
5) In a sterile hood, place 10mL of 10xHBSS in the tube that contains the enzyme.
6) Place 2mL of 10xHBSS in an empty tube.
7) Take 2mL of the enzyme/10xHBSS solution and place it in the tube described in step 6 and mix thoroughly. This is now a 1xHBSS enzyme solution.
8) Take one glass pipette and halve the diameter of the exit hole by carefully rotating the pipette over the flame of a Bunsen burner. Attach a squirt bulb to the pipette.
9) Take 2mL of the enzyme solution of step 7 and place it in the tube containing the antenna.
10) Take step 9 and place it in a hot bath (H2O) that is at a temperature of 37°C. Let the tube remain in the bath for 2 minutes.
11) Take 6mL of culture chow and place it in a tube.
12) Take the flamed pipette of step 8 and wet it with the culture chow.
13) Using the wet pipette, tritrate the antennal tissue five to eight times. It is important to tritrate with gentle pressure. This will ensure explant viability. The goal here is to create explants that are of an optimal size (not too large and not too small).
14) Take all the contents of the tritrated vial and transfer to the tube containing the 6mL of culture chow. Allow the explants to settle to the bottom of the tube (~5 minutes).
15) Remove as much liquid as possible from the tube of step 14 and add 6mL of IL-15. Allow the explants to again settle to the bottom (~5 minutes).
16) Remove as much of the IL-15 as possible without disturbing the explants in the tube.
17) Add 100μL of IL-15 per dish that you are going to develop to the tube containing the explants.
18) Add 1μL of glutamine per dish that you are going to develop to the tube containing the explants.
19) Add 100μL of the explant solution to each dish.
20) Remove 50μL of liquid (w/o removing any explants) from each dish that the inhibitor drug is to be added to.

21) Add 50 L of desired drug to the dishes. Make sure that there are at least two control dishes that contain no added drug.
22) Parafilm© the dishes and store at 27°C for two days.
23) Take pictures of explants at desired magnification (suggested magnification: 10x or 20x).
24) Repeat the steps with any drug that is to be analyzed in the process of neurite growth.

III. Results

Isolation and Identification of Insect Glycosphingolipids

Thin Layer Chromatography Rf values

#	Sample	Rf Value
FATTY ACIDS		
1	Palmitic Acid (C_{16}:0)	**0.69**
2	Oleic Acid (C_{18}:1)	**0.73** +/- 0.05
3	Lauric Acid (C_{12}:0)	**0.69**
4	Stearic Acid (C_{18}:0)	**0.69**
SACCHARIDES		
5	D-Mannose	**0.34**
6	D-Galactose	**0.27**
7	D-Lactose	**0.15**
GLYCOSPHINGOLIPIDS & CERAMIDE MOIETIES		
8	Gb4	**0.52** +/- 0.06
9	C_{18} Ceramide	**0.96** +/- 0.02
EXPERIMENTALLY ISOLATED COMPOUNDS		
10	WHT-1-169a (Acidic GSL Portion)	**0.21—0.48—0.6**
11	WHT-1-169b (N/Nz GSL Portion)	**0.36 & 0.41**
12	WHT-1-135a (Post Dialysis)	**0.66**
13	WHT-1-181a (N-GSL Isolation)	**0.47 & 0.75**
14	WHT-1-181b (Nz-GSL Isolation)	**0.68**

Table 1: The TLC Rf values experimentally determined for various compound.

Illustrated in this table are the Rf values for various compounds that are pertinent in the research of glycosphingolipids. The first group, the Fatty Acids, is composed of possible fatty acids that could be attached to the ceramide moiety. The second group, the

Saccharides, is composed of some possible synthesis sugars for glycosphingolipids. The third group, Glycosphingolipids and Ceramide Moieties, consists of the Gb4 standard and a ceramide standard bought from Matreya. The Gb4 played a crucial role in the study because it acted as the glycosphingolipid standard that could be used for comparison with unknown samples. The last group, the experimentally isolated compounds, consists of samples that were isolated from the Manduca Sexta using the methods discussed in the Methods section of this paper. The best isolation occurred with WHT-1-169a. This sample, according to experimental protocol, was the acidic glycosphingolipids that were isolated from the Manduca Sexta. This solid, obtained from lyophilizing the methanol layer, was dissolved and placed on a Sephadex column. It was then eluted through. The neutral (N) and zwitterionic (Nz) glycosphingolipids eluted of the column first, while the acidic glycosphingolipids remained ionically bound to the diethylamine portion off the Sephadex column. The acidic glycosphingolipids were the washed off the column. Both the N/Nz and acidic GSL containing solutions were rotovapped down to optimal purity and prepared for mass spectrometry analysis. The N/Nz solid that resulted had a brown coloration to it. This may indicate the presence of some impurity. The acidic solid compound was white in color, making it more likely to be purer than the N/Nz extracts. The N/Nz extract was then separated on a silica column. Using a 64: 24: 4 CHCl3: CH3OH: H2O solvent the neutral glycosphingolipids were first eluted off. Then the zwitterionic glycosphingolipids were eluted off the column with the use of a 10: 70: 20 CHCl3: CH3OH: H2O solvent (**15**). Both the neutral and zwitterionic solutions were rotovapped down. The zwitterionic solution resulted in a white powder and the neutral

yielded a brown residue. All three samples were then submitted for mass spectrometry analysis.

Mass Spectrometry Analysis

The results that are conclusive enough to be discussed are those pertaining to the isolated acidic and zwitterionic arthrosides. The acidic compound, sample WHT-1-169b gave a very promising mass spectrometry analysis read out (See Appendices 1.0-1.3). It shows a few high mass peaks and multiple lower mass peaks. These peaks indicate the presence of what appears to be a multi-glycosylated glycosphingolipid. When analyzing the mass spectrometry readouts, it is seen that there are some surprisingly high mass peaks that appear.

The neutral glycosphingolipid extract had promising results as well. The neutral compound's experimental label was WHT-1-181a. The mass spectrometry results indicated that there was a high possibility that this sample contained a multi-glycosylated glycolipid. The two forms of mass spectrometry being shown here are matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). The MALDI results (see Appendices 2.0-2.7) indicate mass peaks going up to the very high range of 2500m/z. Other than a multi-glycosylated, this may indicate some form of agglomeration of the matrix or fragments of the original compound. The ESI readings gave more comprehensible and reliable readings of the contents in the zwitterionic glycosphingolipid-containing compound.

Pathway Perturbation Results

The effects of four varying ceramide analogs were tested on the neurite growth of antennal epithelial explants. The three drugs tested here were D-PPMP, D-PDMP, and L-PDMP. The effects are apparent: an increased level in the D-threo-ceramide analog concentration leads to a decrease in neural processes growth. In contrast: an increased level of L-threo-ceramide analogs (L-threo-PDMP) caused an increase in the neurite growth of the antennal explants. This is apparent from the pictures taken of the explants that had been administered with each of the drugs.

Table of Ceramide Analog Dosage Concentrations

Ceramide Analog	**Dose Concentrations Administered**
D-PPMP	0μM, 2μM, 10μM, and 20μM
D-PDMP	0μM, 2μM, 10μM, and 20μM
L-PDMP	0μM, 2μM, 5μM, and 20μM

Table 2: This table illustrates the dosage that was administered to the antennal explants.

For the D-PPMP and D-PDMP the most minimal neurite outgrowth occurred at the 20 μM and continued to increase growth as the dosage concentration was decreased. Following this trend one could see that maximum outgrowth was achieved with the control samples, due to the fact that no inhibitory drugs were added. For L-PDMP the opposite observation was made. The maximum neurite growth was achieved with the 20μM drug dosage.

IV. Discussion

Isolation and Identification of Glycosphingolipids from Manduca Sexta

The results obtained from the attempts to isolate glycosphingolipids from the Manduca Sexta were successful. As the data shows, the glycosphingolipids that were obtained were the neutral and acidic glycolipids. The acidic isolates are the arthrosides. They have similar functions to gangliosides except they do not contain the sialic acid group. A reliable indicator that was used was the thin layer chromatography. Based of the Rf value that was obtained from an experimental sample, it could be decided whether the techniques being used were performed in the desired manner. In other words, the TLC method is a great rapid assessment and progress indicator towards that isolation of glycosphingolipids. A glycosphingolipid is indicated if the Rf value of the compound isolated using the presented methods is 0.4-0.6. The larger the sugar moiety is on the glycosphingolipid, the lower the Rf value is going to be. The two suspected glycosphingolipids extracts, the acidic (WHT-1-169b) and the zwitterionic (WHT-1-181a), have respective Rf values of 0.21—0.48—0.6 and 0.68. The 0.21—0.48—0.6 Rf value of the acidic extract indicates that it is likely to contain a glycosphingolipid. The reason that there are three values is because there were three different spots present of the TLC plate. The two spots that are intriguing to this research are the ones corresponding to the 0.48 and 0.6 Rf values. It can be hypothetically assumed from these values that there are two glycoconjugates, of which the one with the 0.48 Rf value is more polar than the one with the 0.6 Rf value. This increased polarity could be due to a larger sugar

moiety. However, the 0.6 Rf value component seems to be in the closest range with the Gb4 standard. For the zwitterionic isolate, the Rf value of 0.68 seems to indicate that the compound is pretty nonpoplar since its value is similar to that of a fatty acid. However when observing the mass spectra of WHT-1-181a, one realizes that it appears to contain a glycosphingolipid.

The mass spectra analysis is a key way to determine if there is any desired compound in your unknown sample and what the mass is of that unknown. The two techniques used, MALDI and ESI, gave the best results with the best resolution. In order to commence analysis of these spectra one must first identify the core peak and then determine what that core peak consists of by identifying its fragmentation peaks. In the spectral analysis of the acidic isolate (WHT-1-169b) the core peak was determined to be at 1110.3m/z and then the fragmentation peaks are seen at intervals in the lower m/z range. The same with the zwitterionic isolate (WHT-1-181a). Here one observes that the core peak appears to be at 1346m/z. Here again, one notices the periodic decline of the m/z of the fragmentation peaks. These observations are very characteristic of glycosphingolipids.

Pathway Perturbation

It is observed that the administration of the D-threo-ceramide analogs has definite inhibitory effects on the biosynthesis of glycosphingolipids. Due to this biosynthesis inhibition, the neurite growth decreased as the dosage concentration increased. This does not prove the exact role of glycosphingolipids in the development of neuronal cells. However, it does show that glycosphingolipids are, in some way, involved in the process

of neurite growth. One interesting observation was that when the L-threo-ceramide analogs were administered to growing neuronal explants, the effect was the opposite of the D-enantiomers. An increase in the concentration of the L-enantiomer drug led to an increase in neurite growth. The D-threo-drugs are known to inhibit the glycosyl transferases, however, this is known to probably be untrue for the L- threo-drugs. The L-threo drugs' method of activation is not entirely understood. They probably affect sphingosine, ceramide or glycosphingolipid processing enzyme (2). Of the drugs that were tested here the most effective inhibitor was the D-PPPP.

V. Conclusion

It is conclusive from the data presented here that acidic and zwitterionic glycosphingolipids were isolated. This is proven with the combination of mass spectrometry and thin layer chromatography. The neutral portion of the GSL extraction could not be identified or purified from its contaminated extraction form, therefore, hindering any work to be done on it. The structures of these isolates have been tentatively determined. For the perturbation pathway of the GSLs it was determined that they do participate in some manner in the growth of neural processes. The inhibition of GSL biosynthesis by the ceramide analogs lead to the determination that glycosphingolipids have some sort of control over the outgrowth of these dendrites.

VI. Future Goals

The future goals of the project are to further solidify the structure identification of the isolates obtained from the *Manduca Sexta*. This will be done with the use of further mass spectrometry techniques, gas chromatography, and other extraction methods that are set to a larger scale. Also a protocol for 2-dimensional NMR will be devised. For the GSL pathway perturbation experimentation, the administration of various other ceramide analogs will be carried out. Also, the effects of stereo selectively for varying enantiomers of the ceramide analogs will be further investigated.

Legend

GSL --Glycoshpingolipid

N -- netural

Nz -- zwitterionic

MALDI-- Matrix Assisted Laser Desorption Ionization

ESI -- ElectroSpray Ionization

CH3OH--Methanol

CHCl3 --Chloroform

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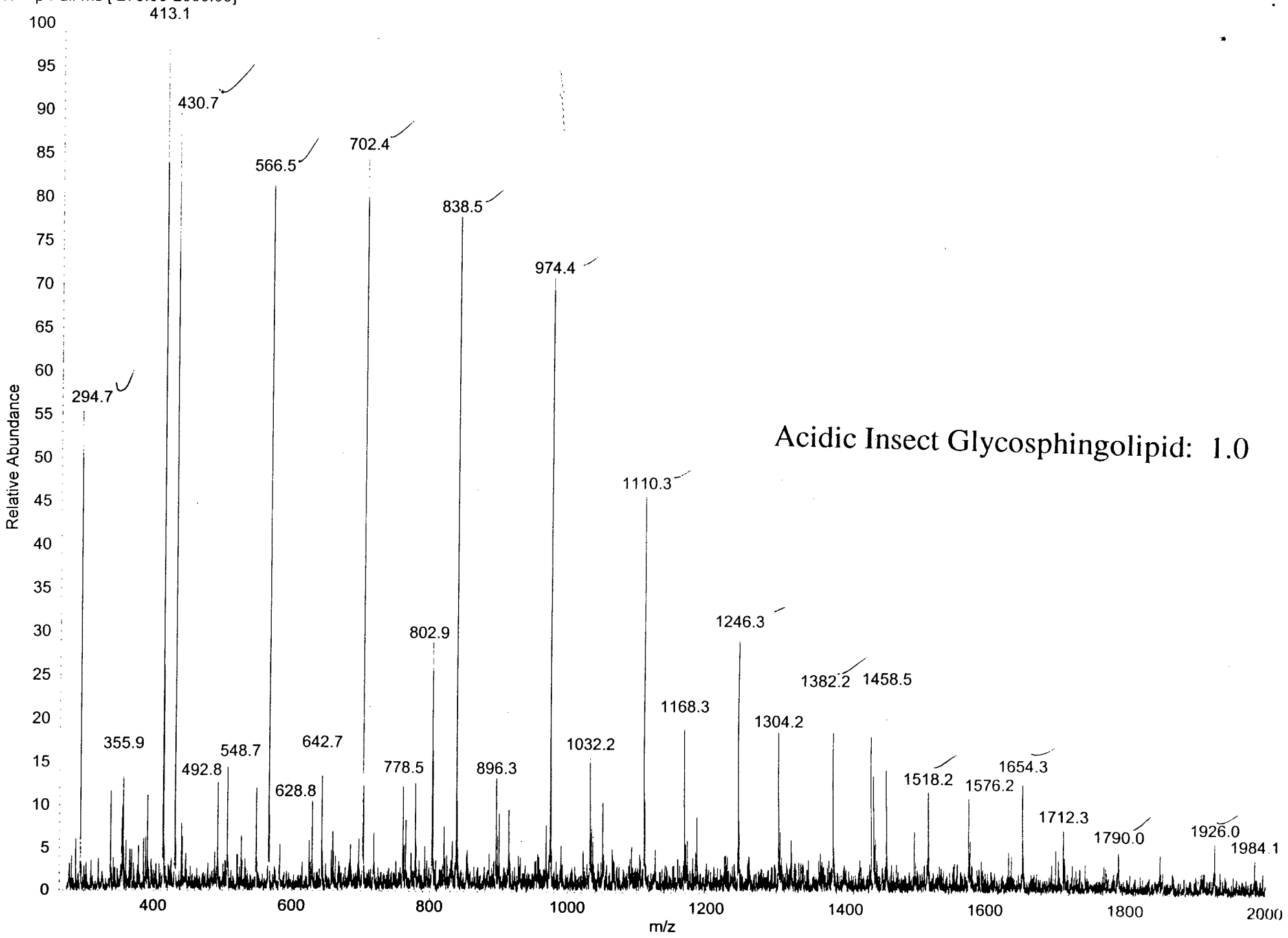
08/01/00 10:09:04

WHT-I-169b



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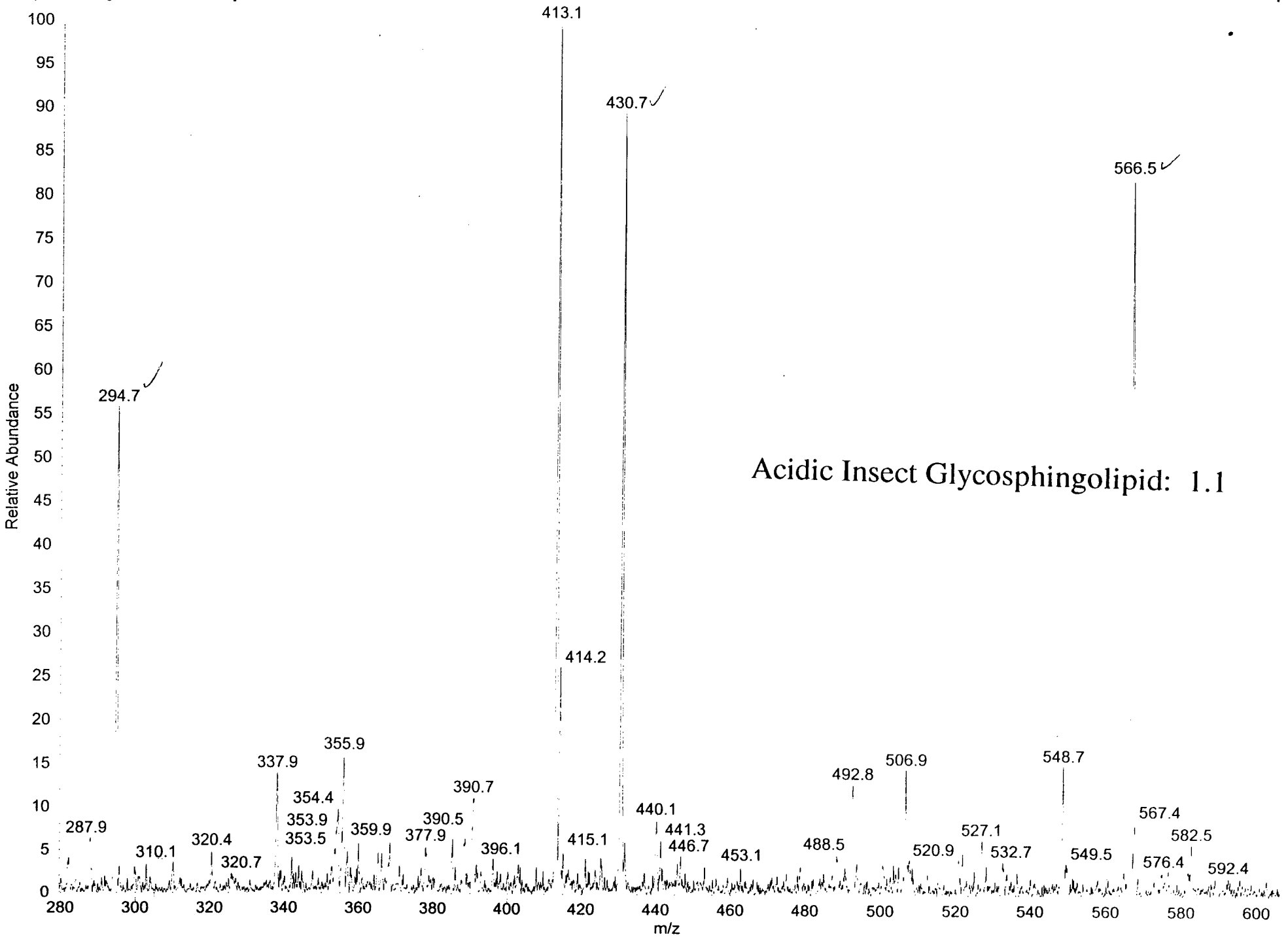
WHT-I-169b



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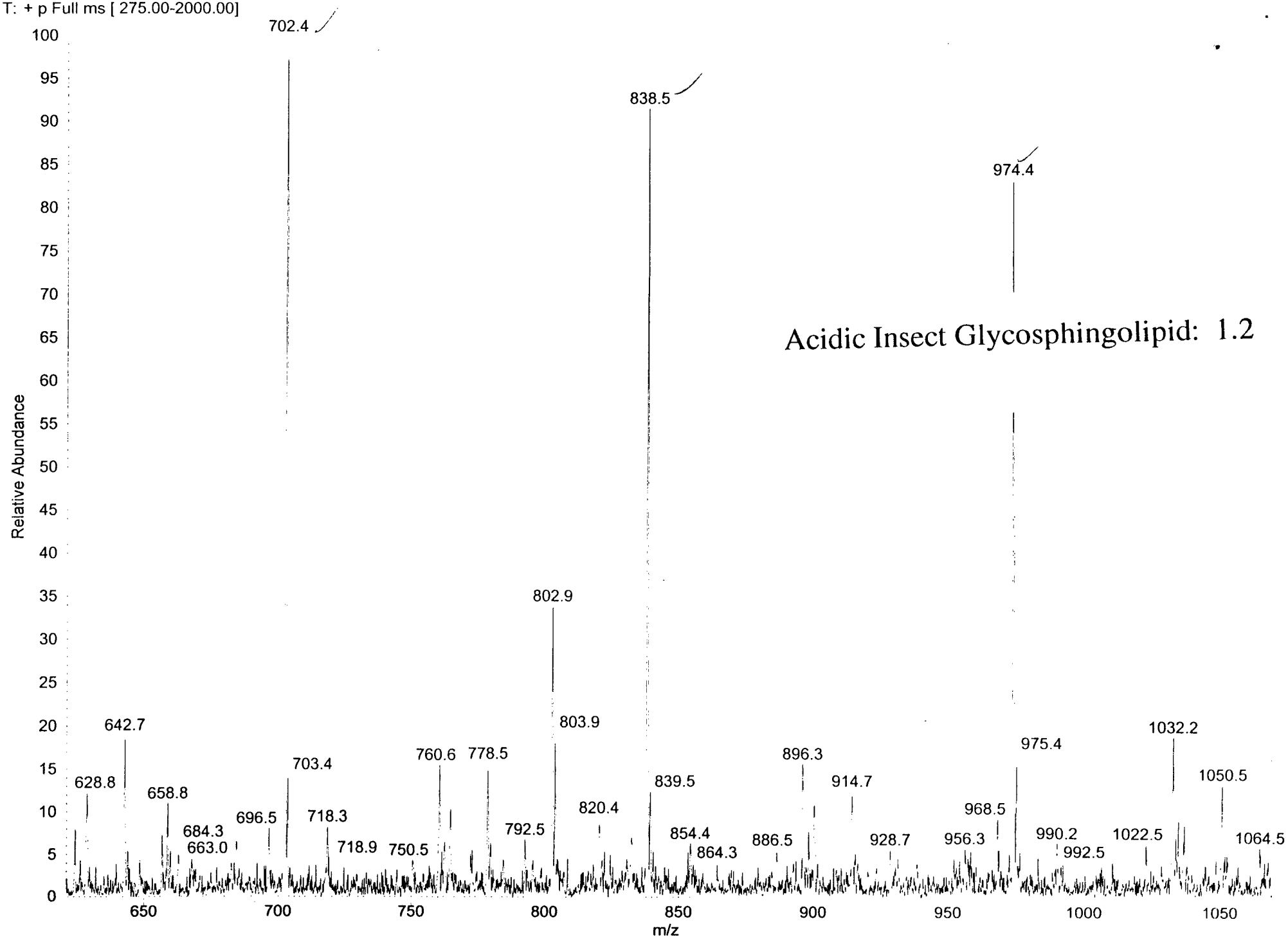

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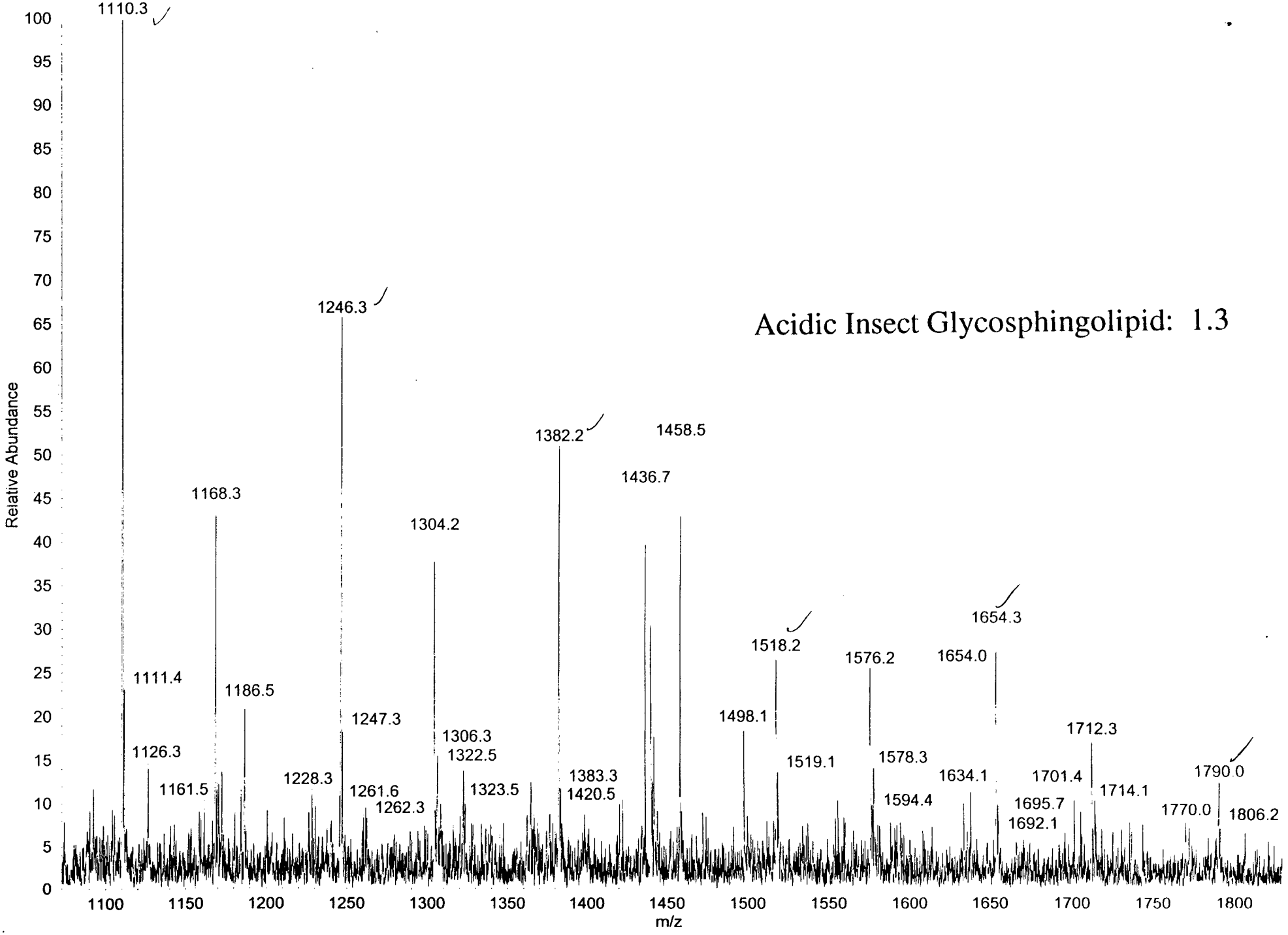
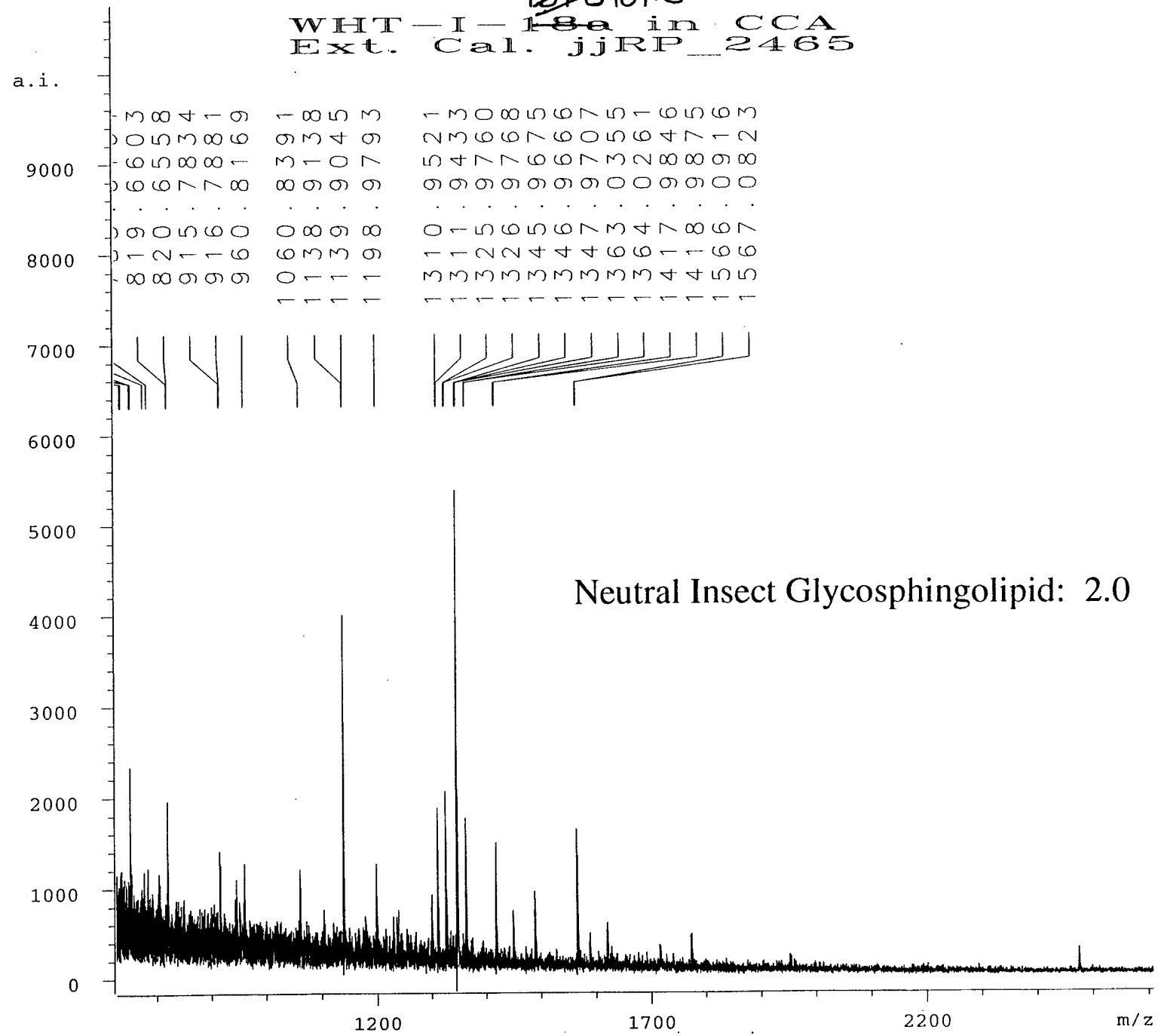


18b 18a

WHT—I—~~18a~~ in CCA
Ext. Cal. jjRP_2465



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Uis2	16.80 [kV]
Uref1	21.00 [kV]
Ulens	8.99 [kV]
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UdetR	1.70 [kV]
Udef1	2.00 [kV]
REPHZ	3.00 [Hz]
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ML2	382.840
ML3	0.000
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LLNSBND	no
UIS2BND	no
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DPMASS	500.00 [Da]
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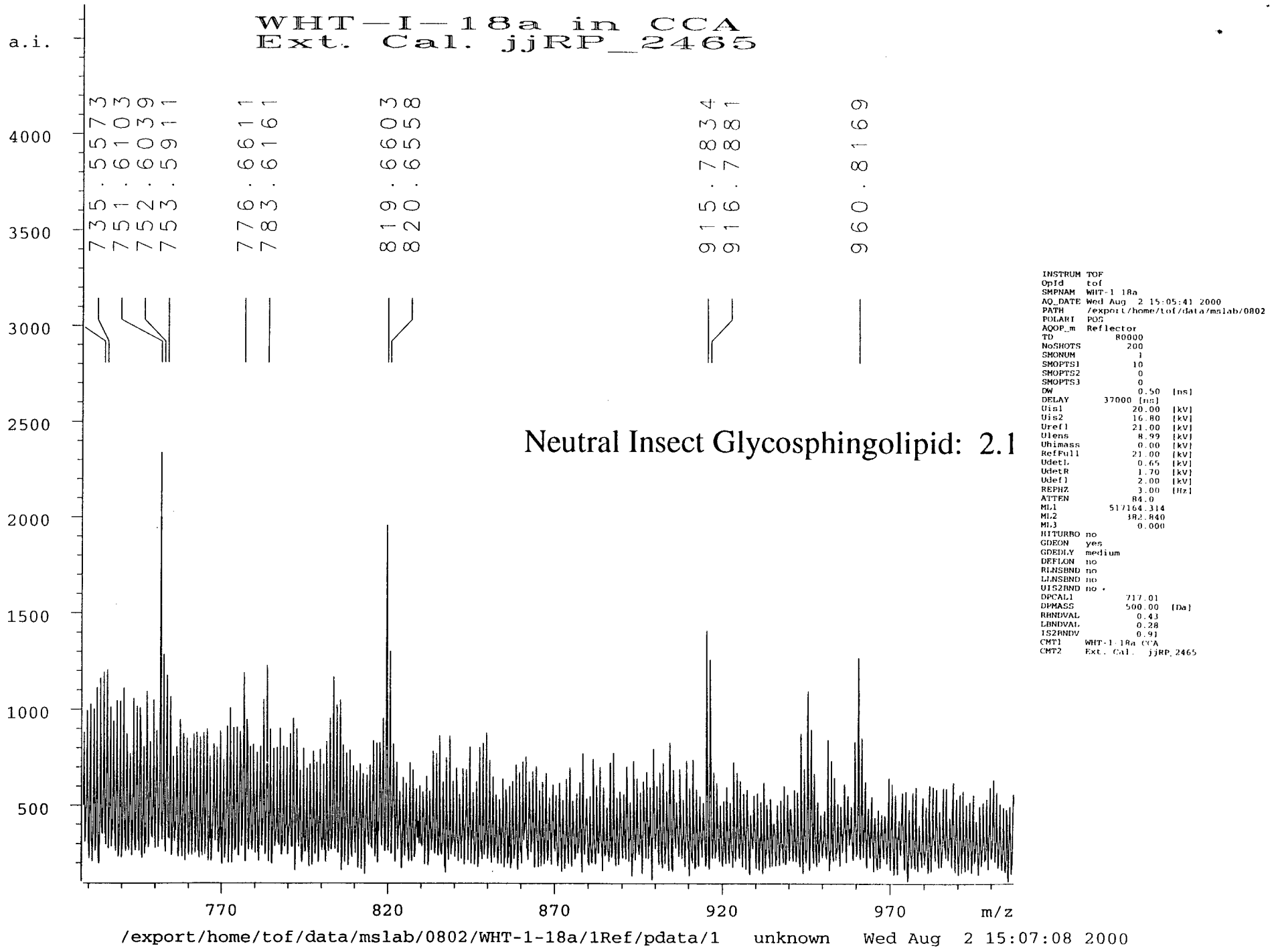
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500
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752.60391
753.59111
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783.61611
819.66603
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Uis2 16.80 [kV]
Uref1 21.00 [kV]
Ulens 8.99 [kV]
Uhimass 0.00 [kV]
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ML2 382.840
ML3 0.000
HITURBO no
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GDEDLY medium
DEFLON no
RLNSBND no
LLNSBND no
UIS2BND no
DPCAL1 717.01
DPMASS 500.00 [Da]
RBNDVAL 0.43
LBNDVAL 0.28
IS2BNDV 0.91
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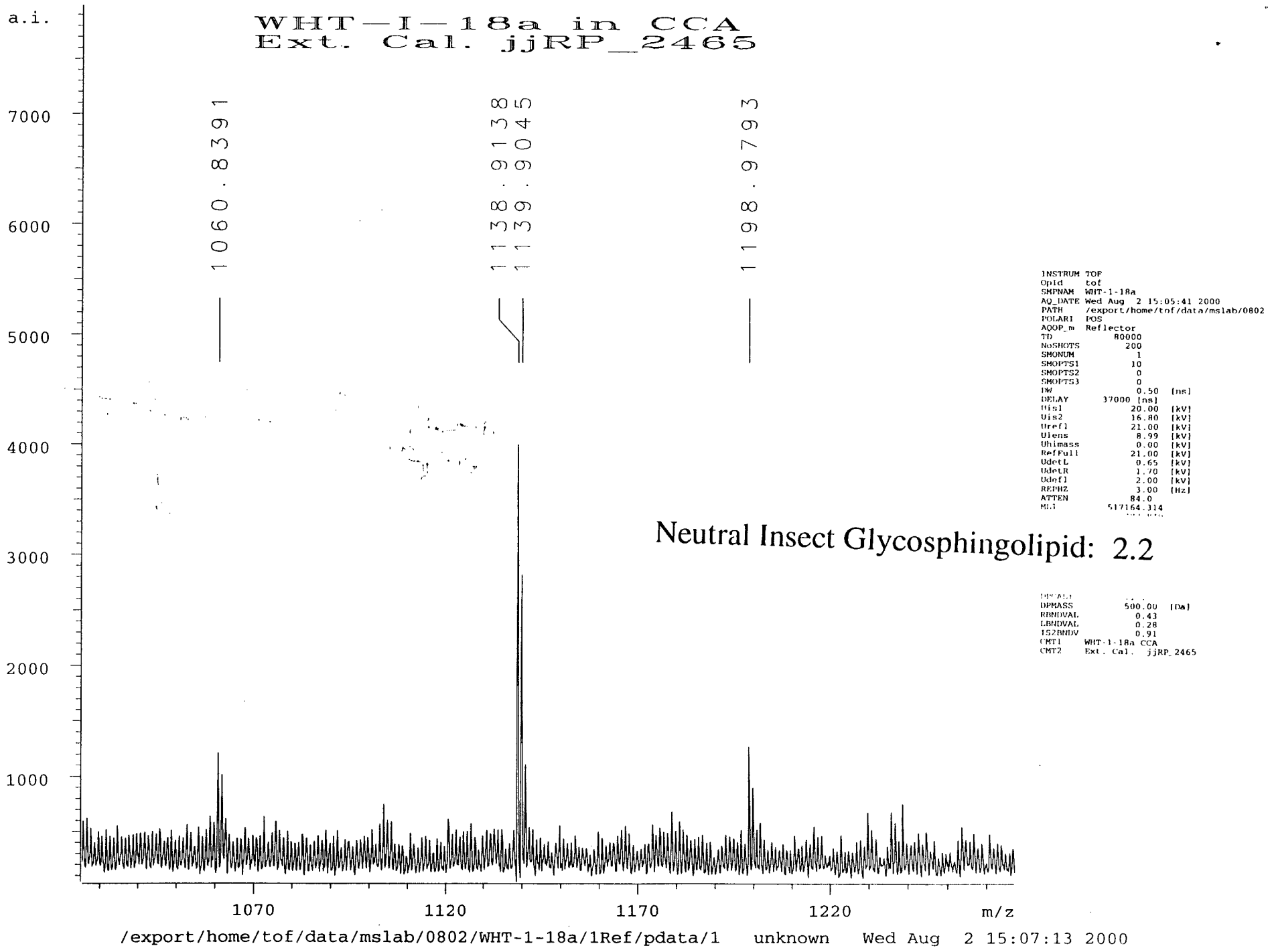

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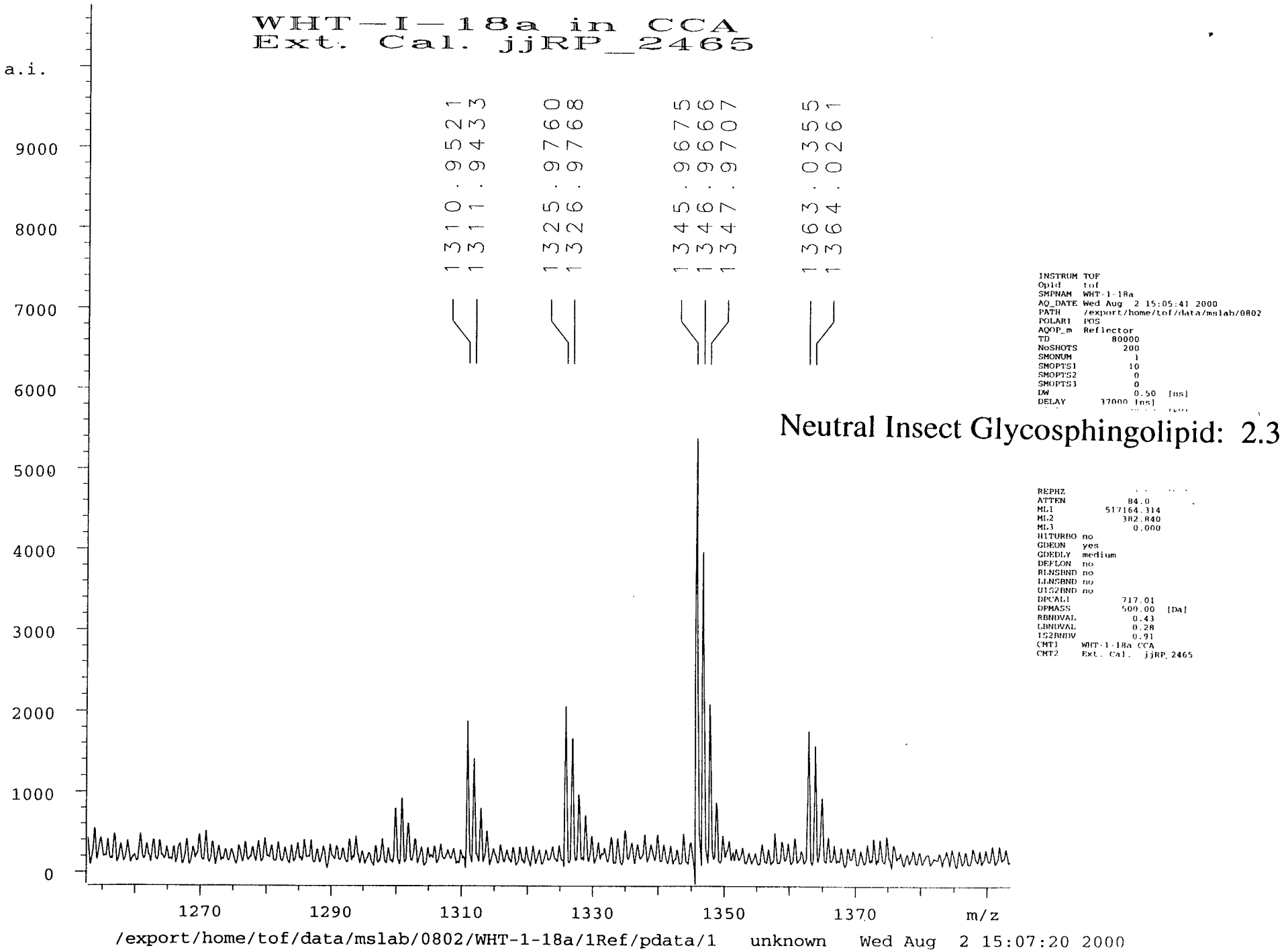

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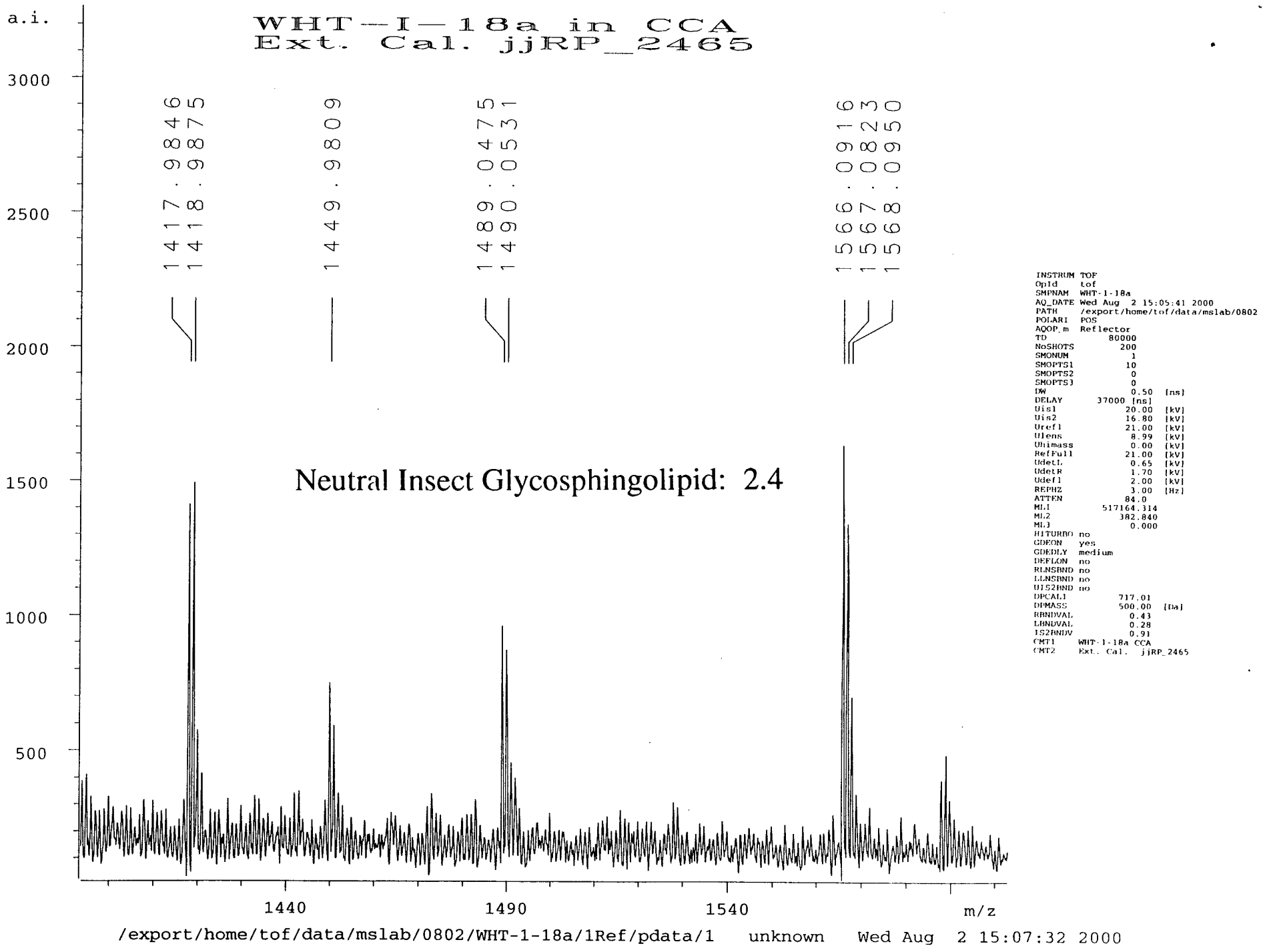
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1566.0916
1567.0823
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Neutral Insect Glycosphingolipid: 2.4
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1490
1540
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Udef1 2.00 [kV]
REPHZ 3.00 [Hz]
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ML3 0.000
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DEFLON no
RLNSBND no
LLNSBND no
UIS2BND no
DPCAL1 717.01
DPMASS 500.00 [Da]
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LBNDVAL 0.28
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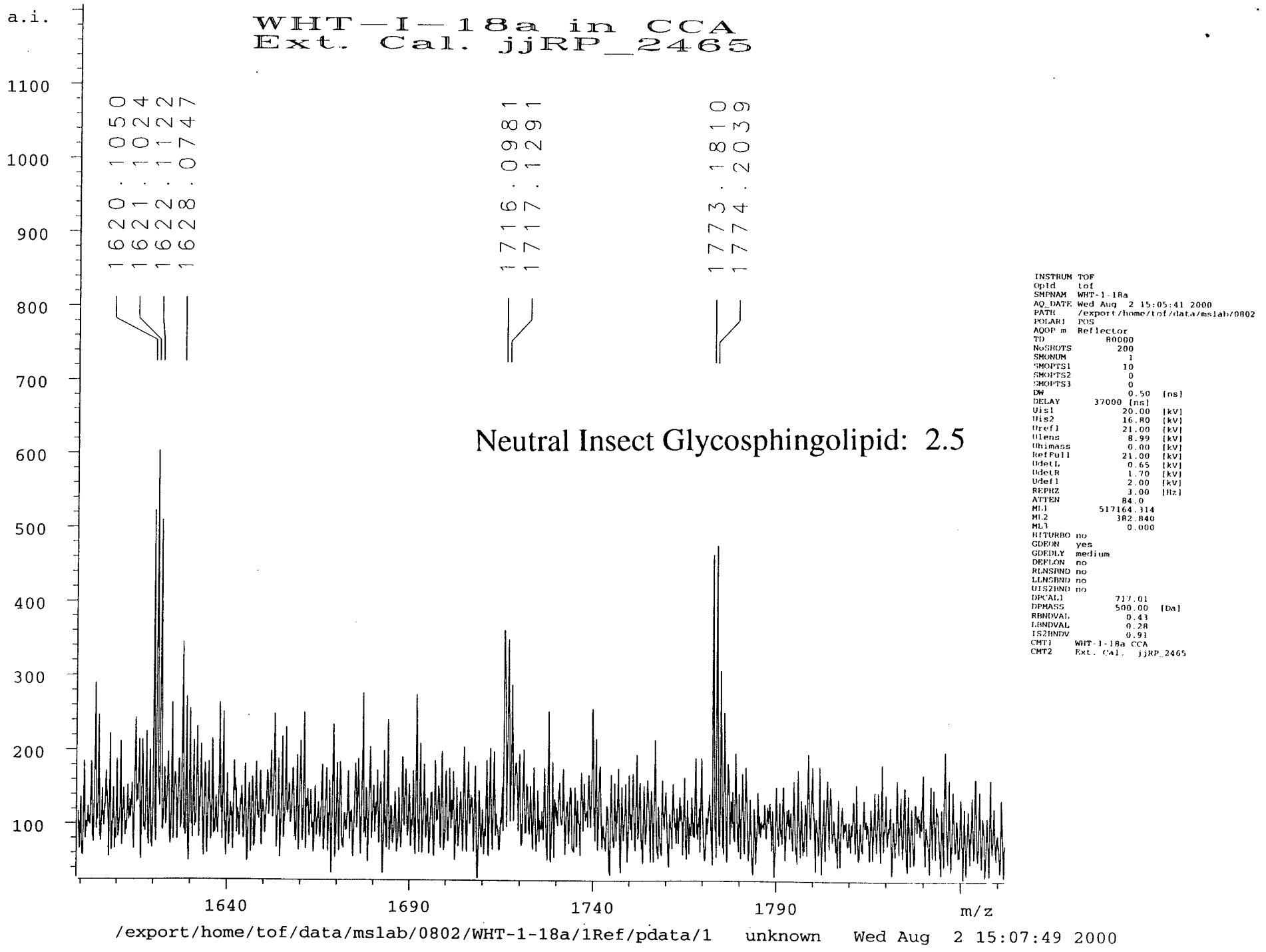
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Ext. Cal. jjRP_2465
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UdetL 0.65 [kV]
UdetR 1.70 [kV]
Udef1 2.00 [kV]
REPHZ 3.00 [Hz]
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ML2 382.840
ML3 0.000
RITURBO no
GDEON yes
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RLNSBND no
LLNSBND no
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DPCAL1 717.01
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RBNDVAL 0.43
LBNDVAL 0.28
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1740
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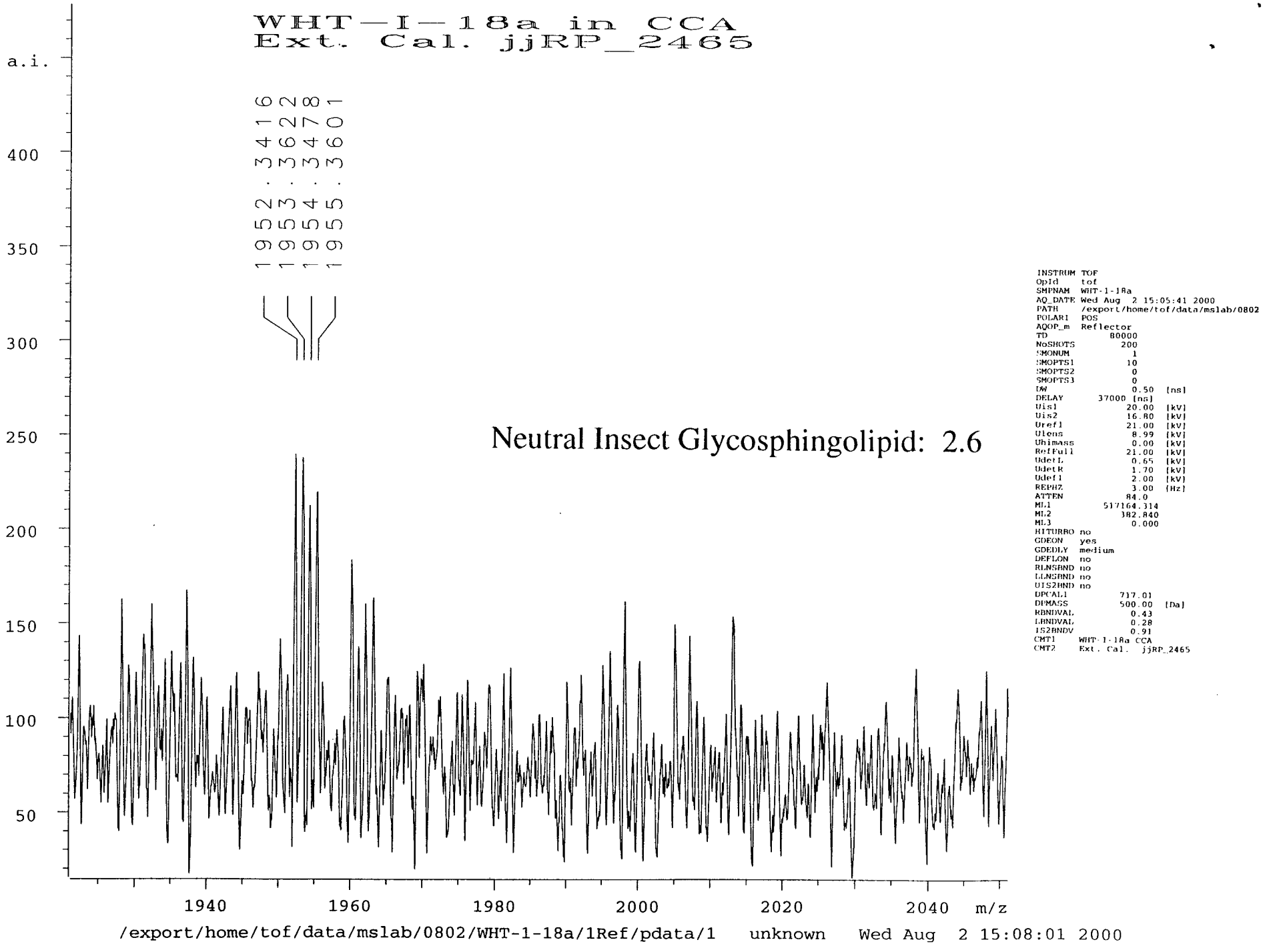
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150
100
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SMOPTS3 0
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Vref1 21.00 [kV]
Vlens 8.99 [kV]
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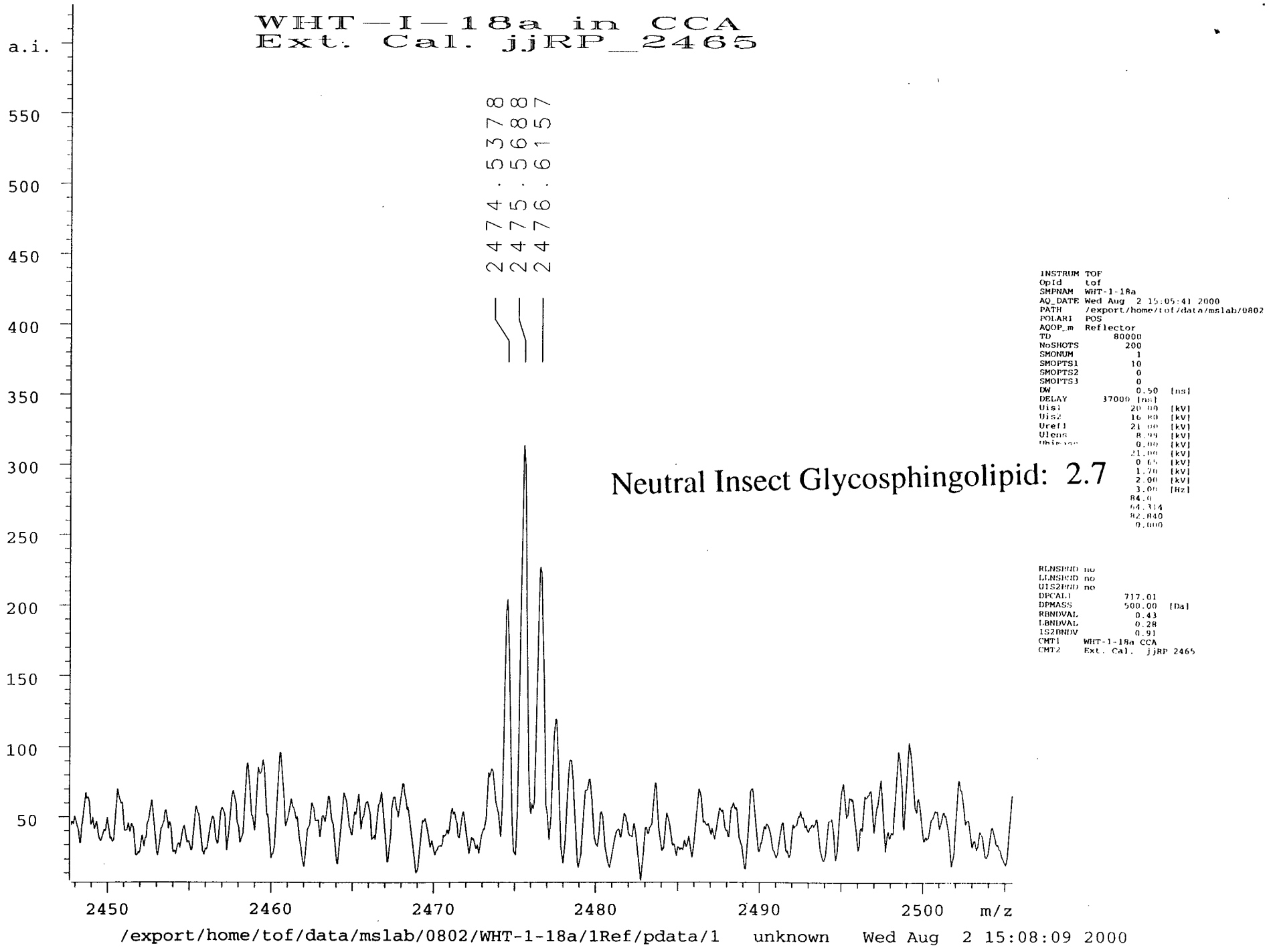
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Uref1 21.00 [kV]
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21.00 [kV]
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IS2BNDV 0.91
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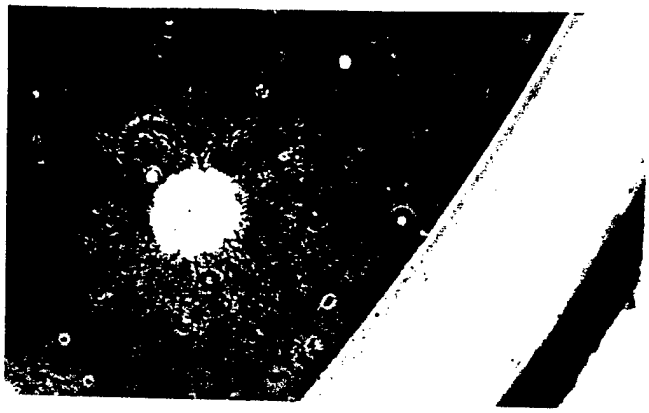
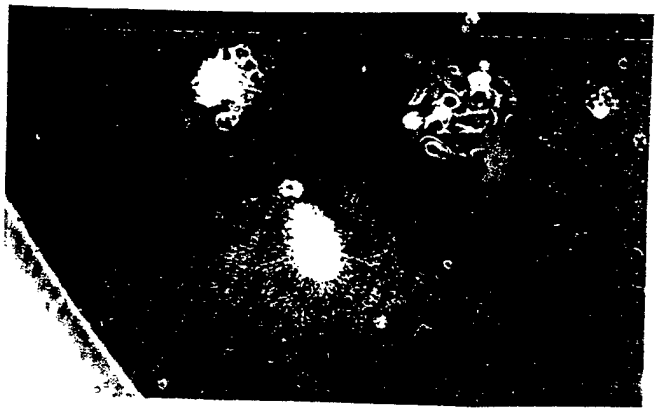
Illustration of Inicreasing Dosage Concentration of L-Threo-Drugs on Neurite Growth

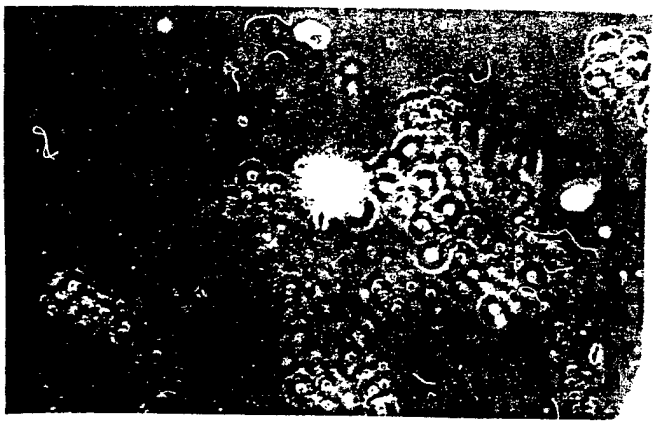
0μM PPMP



4μM PPMP



40μM PPMP



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(NEE WIJAYARATNE)

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E-MAIL: THUSITHA@CHEM.CMB.AC.LK
(Work phone) 01-503367

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PERSONAL INFORMATION

- Marital status: Married
- Nationality: Sri Lankan
- Age: 36 years

EDUCATION

1988 - 1993
University of Arizona,
Tucson, Arizona, USA.
- Ph. D. (Organic Chemistry)
- Thesis title, *"Part I: 2-deoxy-C-Glycofuranosides from D-glucose. Part II: An Approach to N-actylneuraminic acid from carbohydrate precursors"*

1983 - 1986
University of Colombo
Colombo 3, Sri Lanka.
- B.Sc. (Chemistry Special)

PROFESSIONAL EXPERIENCE

August 1999 - at present
Department of Chemistry, University of Colombo,
Colombo 3, Sri Lanka.
- Senior Lecturer: grade 1

August 1993 – July 1999
Department of Chemistry, University of Colombo,
Colombo 3, Sri Lanka.
- Senior Lecturer: grade 11

June 1991 - July 1993
Department of Chemistry, University of Arizona,
Tucson, AZ 85721, USA.
- Internship in materials characterization programme, NMR facility

August 1988 - May 1991
Department of Chemistry, University of Arizona,
Tucson, AZ 85721, USA.
•Teaching Assistant

January 1987 - July 1988
Department of Chemistry, University of Colombo,
Colombo 3, Sri Lanka.
•Assistant lecturer

ADDITIONAL PROFESSIONAL ACTIVITIES

1999 - 2000
Faculty of Medicine, University of Colombo, Sri Lanka.
•Conducted lectures for the Diploma i •Pharmacy.

1998
Department of Chemistry, University of Ruhuna, Sri Lanka.
•Visiting Lecturer

1995 -1997
Department of Chemical Engineering, University of Moratuwa,
Moratuwa, Sri Lanka.
•Visiting Lecturer

1996 -1997
Department of Botany, University of Colombo,
Colombo 3, Sri Lanka.
•Conducted lectures for M. Sc. Course i •plant cell and tissue culture.

1995 -1999
Institute of Chemistry,
120/10, Vidya Mandiraya, Colombo 7, Sri Lanka.
•Visiting Lecturer

COMMUNICATIONS AND PUBLICATIONS

1. Anti-bacterial activities of Volvariella volvacea, W.A.S.W. Perera, D.T.U. Abeytunga, R.L.C. Wijesundera *J. Natn. Sci. Foundation,* Sri Lanka (submitted in **1999**).

2. Characterization and assessment of anti-fungal activity of chemically modified berberine, D.T.U. Abeytunga, B.N.Pathiratne, W.M.S.R. Ratnayaka *J. Natn. Sci. Foundation, Sri Lanka* (accepted in **2001**).

3. Characterization and assessment of anti-fungal activity of chemically modified berberine, D.T.U. Abeytunga, B.N.Pathiratne, W.M.S.R. Ratnayaka *J. Natn. Sci. Foundation, Sri Lanka* (accepted in **2001**).

4. Screening of the methanol extract of Pleurotus ostreatus for antibacterial and antifungal activity, D.E.Jayawardena, D.T.U. Abeytunga, R.L.C. Wijesundera *Abstracts, Annual Sessions, Faculty of Science, University of Colombo,* 1, 2, **2000**

5. Isolation and identification of fungi from mushroom composts and evaluation of their biological activity, Ramani Wickremasinghe, Krishanthi Abeywickrama, D.T.U. Abeytunga *J. Natn. Sci. Foundation, Sri Lanka,* 27(1), 29, **1999**.

6. Isolation and identification of hexoses and nucleosides from Volvariella volvaceae, W.A.S.W. Perera, D.T.U. Abeytunga Chemistry in Sri Lanka, 16(1), 9, **1999**

7. Activity of some aromatic acids against Rhizoctonia solani, D.T.U. Abeytunga, T.E.M. Peiris, V. Jayasinghe, R.L.C. Wijesundera *Sri Lanka Association for the Advancement of Science,* 54th Annual Session, Dec. **1998**.

8. Structure-antibacterial activity of some aromatic acids, D.T.U. Abeytunga, T.E.M. Peiris, R.L.C. Wijesundera *J. Natn. Sci. Coun. Sri Lanka,* 26(2), 133, **1998**.

9. Furans from the culture filtrate of Trichoderma viridae, D.T.U. Wijayaratne, W.A.S.W. Perera *Sri Lanka Association for the Advancement of Science,* 53rd Annual Session, Dec. 1997.

10. Antifungal activity of some berberine derivatives, D.T.U. Wijayaratne, C.T. Sirimanne, H.G. Jayatillake, I.W.A.D.P. Palipana *Chemistry in Sri Lanka,* 14(1), 9, **1997**.

11. Antibacterial activity of Piperine and its derivatives, D.T.U Wijayaratne, D. Herath, R.L.C. Wijesundera *Chemistry in Sri Lanka,* 14(1), 17, **1997**.

12. Isolation of an antifungal aza-sugar from Aspergillus fumigatus, D.T.U. Wijayaratne, K.P.Abeywickrama, P.D.F. Chandani, R. Wickramasinge *Sri Lanka Association for the Advancement of Science,* 52nd Annual Session, Nov. **1996**.

13. Stereochemistry of 4,5-Dihydroxy a santonin and structure of a New Santonin Oxidation Product, S.K.Paknikar, B.L.Malik, R.B.Bates, S. Caldera and T.V. Wijayaratne *Tetrahedron Lett.,* 35, 8117, **1994**.

14. β-Alkoxy-Schiff base-oxazolidine tautomerism; solid-state structure of N-Diphenylmethylene-L-threonine methyl ester. Thusitha Wijayaratne, Nathan Collins, Yshun Li, Michael A. Bruck and Robin Polt *Acta Crystalogr.,* B49, 316, **1993**.

15. Erythrose sesqui-acetals as electrophiles. 2-deoxy-C-nucleosides from D-glucose. Robin Polt and Thusitha Wijayaratne *Tetrahedron Lett.,* 32, 4831, **1991**.

16. Plasma concentrations of chloroquine and its metabolite after oral administration in uncomplicated malaria., K. Weerasuriya, M.P.D. Mahindaratne, P.L.D.S. Perera, D.T.U. Wijayaratne, L.M.V. Tillekeratne *European Journal of Clinical Pharmacology,* 36, A122, **1989**.

SYMPOSIUMS / WORKSHOPS ATTENDED

1. " New synthetic methodology for 2-deoxy-C-Nucleoside Analogs", T. Wijayaratne, R. Polt, 9th Biennial Carl S. Marvel symposium, 11-13 March 1991, Tucson, Arizona, USA. (Poster)

2. "An antifungal bis-azasugar form Aspergillus fumigatus", D.T.U. Wijayaratne, K.P. Abeywickrama, P.D.F. Chandani, Symposium on Bioactive Natural Products, 11-15 November 1996, Kandy, Sri Lanka.

3. Attended a "Workshop on NMR spectroscopy", 15-20 September 1998, University of Peradeniya, Kandy, Sri Lanka.

PROFESSIONAL MEMBERSHIPS

1993 (Life member)
- •Membership of Sri Lanka Association for the Advancement of Science (SLAAS)
- •1997, committee member, Chemical science section, SLAAS

1993 (Life member)
- •Membership of Institute of Chemistry, Ceylon
- •1997, Committee member, Interschool chemistry quiz committee, Institute of Chemistry

CERTIFICATES/AWARDS RECEIVED

1992
University of Arizona, Tucson, Arizona, USA
- •$800 from the Graduate student research fund for research

1991
University of Arizona, Tucson, Arizona, USA
- •Certificate of appreciation (For assisting graduate assistant teaching orientation)

EXTRACURRICULAR ACTIVITIES

1989-1993
Medical School, University of Arizona, Tucson, AZ 85721, USA
- •Participated in the OSCE evaluation programme

PROFESSIONAL REFERENCES

1. Prof. Robin Polt, Dept. of Chemistry, University of Arizona, Tucson, AZ 85721, USA.
2. Prof. R.B. Bates, Dept. of Chemistry, University of Arizona, Tucson, AZ 85721, USA.
3. Prof. E.D. de Silva, Dept. of Chemistry, University of Colombo, Colombo 3, Sri Lanka.
4. Prof. H.D. Gunawardane, Dept. of Chemistry, University of Colombo, Colombo 3, Sri Lanka.
5. Dr. S. Hewage, Head, Dept. of Chemistry, University of Colombo, Colombo 3, Sri Lanka.